Microbial enrichment of torrefied grass fibers – a novel ingredient of potting soil

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To mama, papa, uncle Stoicho and Gergana
На мама, татко, чичо Стойчо и Гергана

Contents

CHAPTER 1

General Introduction

Torrefied grass fibers as an alternative for peat in potting mixes

Potting mixes used for plant growth are commonly composed of peat. Sphagnum and sedge peat are the most common types of peat used in horticultural growing media. These are taken from natural peat bogs, ecosystems that are highly fragile with great ecological value [45]. Most peat bogs have developed naturally for thousands of years and are currently being threatened. For instance, substantial natural peat lands or bogs have been lost in Europe, mostly in the Netherlands, Poland, Germany, the UK and Ireland. For instance, in the UK and Ireland more than 80% of peat bogs have been lost [7]. An intensive search for peat substitutes is thus needed, on the one hand because of the limitation of peat in the near future, and on the other hand because of environmental constraints [1]. Since the late 1970s, there has been a worldwide search for peat substitutes [95, 98]. Recently, increased attention has been given to the recycling of solid organic waste for use as constituents in substrates for ornamental pot plant production [2, 27, 42, 123].

Alternatives for peat should ideally match the characteristics that make peat such an excellent growth substrate for plants. These characteristics are:

- Absence of plant diseases, pests and weeds
- Excellent air/water balance and a high water-holding capacity
- Resistance to physical and microbial decomposition to prevent shrinkage in the pot
- Slightly acid pH, with pH 5.5-6.5 as optimal value
- High buffering capacity for nutrients
- Low electrical conductivity, i.e. low salt content and low nutrient status
- Consistent quality

Different compounds have been investigated as peat substitutes. Wood fiber [38], coconut coir dust [27], pine bark [127], compost from different sources [39, 94, 132], cotton gin trash [87] and dried sewage sludge [52, 84, 96] have all been considered for this purpose. None of these compounds has been completely satisfying, though, and main issues have been that most peat substitutes have a lower water holding capacity than peat or a too high salt content.

Recently, a new potential peat replacement has been suggested, namely thermally treated (torrefied) grass fibers (TGF) (fig. 1). Grass grows worldwide and it represents a renewable substrate, in contrast to peat, and hence it may constitute an environmentally friendly substitute for peat in potting media. Untreated grass is too degradable. It also may contain viable weed seeds and plant pathogens. However, by exposing dried grass fibers to

a treatment of 240°C for one or more hours under conditions of low oxygen (a process known as torrefaction), the fibers are transformed into a stable matrix which is fairly protected against biological decay (J.J.M.H. Ketelaars, unpublished data). A solid product with low moisture content is thus obtained. The torrefaction can be characterized as “mild pyrolysis”, in which removal of gaseous compounds and formation of a solid end product take place. Torrefied grass fibers have excellent water holding capacity; however, a disadvantage of the torrefaction is that it may enhance the phytotoxicity of the material (J.J.M.H. Ketelaars, unpublished data). It is not clear yet which compounds from the torrefied grass fibers cause the phytotoxic effect. Recent data show that particular phenolic compounds (2-methoxyphenol; 2,6-dimethoxyphenol) cause phytotoxicity problems [6, 91, 119]. The release of such phenolic compounds probably arises from lignin depolymerization. The phytotoxic compounds 2-fural aldehyde (furfural), pyrrole-2-carboxaldehyde and furan-2-methanol are also produced. Such products and derivatives (e.g. furfural and hydroxymethyl furfural) are produced by the dehydration of solubilised sugars [13, 71].

Fig 1. Torrefied grass fibers

Colonization of torrefied grass fibers (TGF)

The sterile conditions in the TGF directly following the heat treatment offer ideal circumstances for the introduction of selected microorganisms with desired properties. In fact, TGF can be said to represent a microbiological vacuum, as it theoretically contains no viable cells and the nutrients that are potentially present in it have not yet been used. Thus, TGF offers novel colonisable niches for microbes. How many niches would there be in this substratum and what would constitute a successful and stable microbial consortium in it? Answers to these questions have been lacking, but, given an expected diversity of microhabitats that offer diverse colonization and growth possibilities, it is plausible that

niches will exist that allow the establishment of both *r*- and *K*-selected microorganisms. Thus, if microorganisms with specific desirable properties - like plant growth promotion - colonize such niches in the TGF and establish in these, plants that grow on the substrate might ultimately benefit from the established microbiota. TGF may thus be considered as a novel prospective ingredient for potting soils.

The early stages of colonization of a novel habitat by a microbial community are often characterized by low diversity and evenness values [120]. With time, an increasing number of (pre-existing or newly-formed) niches will be filled, and, given that the early easily-available substrates will become depleted, increasing demands will be placed on the competitive abilities, in each of the emerging niches, of the individual populations that make up the community. As a result, the diversity and evenness levels of the communities in the system may increase [28].

During succession in the conquest of the novel niches, two major groups of microorganisms, representing two growth strategies, may be distinguished, i.e. the copiotrophs and the oligotrophs, also alikened to the so-called *r*- and *K*-strategists [65]. Of these, the *r*-strategists thrive best as primary colonizers, as they have intrinsic high growth rates, allowing them to quickly utilize abundant resources that are available. These microorganisms may be out competed - in a next successional stage - by microbes with a narrower niche width, characterized by slower growth and lower investment in reproduction, such as the *K*-strategists. The general characteristics of *r* and *K*-strategists are summarized in Table 1.

Theoretically, a stable microbial community in TGF will need to consist of a number of both *r*- and *K*- strategists, as this may allow an adequate balance between the different ecophysiological types in the matrix.

Novel niches provided by the TGF system will first be colonized by (a limited number of) *r* strategists (on the basis of their opportunistic character sometimes alikened to microbial “cockroaches”). These organisms may show little substrate or niche specialization and are expected to rapidly utilize different easily-available substrates [28]. Following initial fast growth of *r*-strategists, *K*-strategists may come up. Such organisms are characterized by their capacity to grow under conditions of crowdedness and scarcity of resources, and persist. Thus, stable and mature microbial communities on TGF should ideally comprise microorganisms of diverse ecophysiological types, including *r*- as well as *K*- strategists.

Table 1. Characteristics of *r*- and *K*-selected microorganisms

Selection criterion	*r*-selected species	*K*-selected species
Growth rate	Rapid	Slow or moderate
Substrate utilization	Nutrient-demanding	Moderately nutrient demanding
Utilization efficiency	Relatively low	Relatively high
Diversity of substrate used	Simple, readily available	Able to use diverse, complex materials
Phenotypic plasticity	Polymorphic to monomorphic	Monomorphic
Population dynamics	Explosive, density-independent, non-equilibrium, recolonization, high migratory tendency	Density-dependent by competition, equilibrium dynamics, low migratory tendency
Tolerance to niche overlap	Relatively large	Relatively small

Langer *et al* [65]

Availability and transformation of carbon sources

The types of carbon sources that are initially present in the TGF will determine the microbial community that establishes on the matrix and the microbial interactions within the community. Upon torrefaction, meant to chemically stabilize the substratum, a few broadly and easily-available carbon sources will occur in the TGF. We showed that acetic acid (465.6 mg/kg) and formic acid (791 mg/kg) dominate among these [115]. These compounds can serve as nutrient and energy sources for many microorganisms. As they represent “simple” easily available bound carbon presumably present at different microhabitats in the matrix, it follows those different relationships between the available carbon and TGF colonizers (*r*- and *K*-strategists) will ensue. This suggests that the relative dominance of each ecophysiological type may be dependent on C availability as well as microhabitat conditions. Such microhabitat conditions include factors such as pH, water and oxygen content, as well as the presence of other compounds. Key compounds in the TGF, such as furfural and alike, and phenolics, might thus affect initial colonization. Their removal is therefore required in order to allow full-blown colonization of the TGF.

Microorganisms with the capacity to transform such compounds would have an ecological advantage in colonizing TGF, meanwhile reducing the phytotoxicity of the substrate. Finally, microorganisms that are equipped with plant-beneficial properties would give the microbiologically matured substrate an added value. Plant-growth-promoting bacteria, as well as plant pathogen inhibiting microorganisms, are prime candidates to make part of the microbial consortium.

Can the toxic compounds present in the TGF be reduced by microorganisms? So far, organisms such as *Escherichia coli* strains LYO1 and KO11, *Klebsiella oxytoca* P2 and particular *Enterobacter, Citrobacter, Edwardsiella* and *Proteus* strains [10, 13, 23, 41, 70] as well as the fungi *Coniochaeta ligniaria* [78], *Pleurotus* spp. [118], *Pycnoporus cinnabarinus* and *Coriolopsis rigida* [6] were found to be able to detoxify compounds such as 2-furalaldehyde, furan-2-methanol and 5-hydroxymethyl furfural (5-HMF), as well as some phenolic compounds. These compounds can be found on TGF. Hence, microorganisms able to utilize the toxic compounds in TGF should be isolated and used to make TGF colonization successful. Another important aspect is the C:N ratio in the available substrates present in the TGF, which should ideally be close to 5 - 10 to allow the most optimal nutritional conditions for bacteria [25]. Fungi are reported to have a higher C:N ratio (5–15) than bacteria (3–6) [89, 124]. In general, the in situ C:N ratio will be difficult to measure due to problems in obtaining microhabitat samples without confounding compounds.

Plant-beneficial properties of TGF when used as potting soil

The novel empty niches in the TGF will in principle offer the opportunity to establish a stable microbial community, which encompasses a range of plant-beneficial bacteria. There are different plant-beneficial bacteria, under which a number of plant-growth-promoting and plant pathogen inhibiting (biocontrol) ones. In particular species of the genus *Serratia* encompass many biocontrol agents [54, 85, 97, 110], some of them being prime in situ producers of anti-phytopathogen antibiotics like pyrrolnitrin [31, 69, 75]. Also, *Pseudomonas* strains with antagonistic properties against soil-borne pathogens form an important group of putative biocontrol agents [18, 30, 31]. For instance, particular *P. corrugata* strains are known as key antagonists of the pathogens *Botrytis cinerea[40]*, *Pythium aphanidermatum* and *P. ultimum* [33, 37, 106], *Ralstonia solanacearum biovar* 2 [121], *Fusarium oxysporum* [24, 105] and *Monilinia fructicola* [108]. Other examples of key biocontrol agents include *Paenibacillus polymyxa,* being antagonistic against *Fusarium oxysporum* [20], *Pseudomonas* and *Serratia* spp. - against *Verticillium dahliae* [8] - and *Pseudomonas* spp. against *Rhizoctonia solani* [29]. On the other hand, different rhizosphere and endophytic isolates have been shown to be active as plant-growth

promoting bacteria (PGPR) and the mechanisms involved were found to be diverse [43, 112]. PGPR stimulate plant growth through any of the following mechanisms: (1) by altering the hormone balance in the host plant; (2) by increasing mineral nutrient solubilization; and (3) antagonism towards plant pathogens [26].

Bacterial-fungal interactions

In the establishment of a plant-beneficial microbial community on TGF, the role of particular fungi should also be taken into account. The main reason is that fungi, by their particular ecophysiology (growth) in a matrix, offer particular characteristics that are lacking in many bacteria. Secondly, fungi are known to play key roles in complex natural environments, and they may even provide ecological opportunities for bacteria that occur in their surroundings [58, 125]. On the other hand, bacteria that occur in the same microhabitat as particular fungi may show differential behavior in the face of the fungal component [15, 56]. Most often, though, particular bacteria can benefit from fungi that are locally present, for instance in a soil setting. This may result from the presence of fungal exudates which may serve as exclusive sources of bacterial nutrients [4, 82, 133]. The bacteria that become associated with fungi may even obtain their benefit at the expense of the fungal partner, e.g. via antagonistic or predatory action [17, 46, 56]. Given the particular roles of fungi in microbial communities in complex habitats, it is important to understand bacteria-fungal interactions.

Outline of strategy used to produce microbiologically-colonized TGF

The strategy used to produce microbially colonized plant-beneficial TGF is presented in fig 2. Torrefied grass fibers are prepared following a standard procedure, in which, unfortunately, toxic substances are produced.

TGF and its extract are then inoculated with a suspension of microorganisms obtained from soil, followed by subsequent enrichment procedures that aim at the isolation of microorganisms that are adapted to growth on TGF. Diverse bacterial strains are isolated and subjected to various screens, including growth on (phyto)toxic compounds and a lettuce seed germination assay. Both screens assess their capacity to decrease the level of phytotoxicity in the matrix. A microbial consortium is then established and evaluated for the level of colonization of the TGF. In the light of the colonization data, a suitable mix of particular bacteria and the ascomycetous fungus *Coniochaeta ligniaria* is concocted, allowing persistent colonization of TGF and utilization of toxic compounds. Finally, the quality and potential of the enriched TGF substrate is tested as a substratum to be added to peat in the support of plant growth.

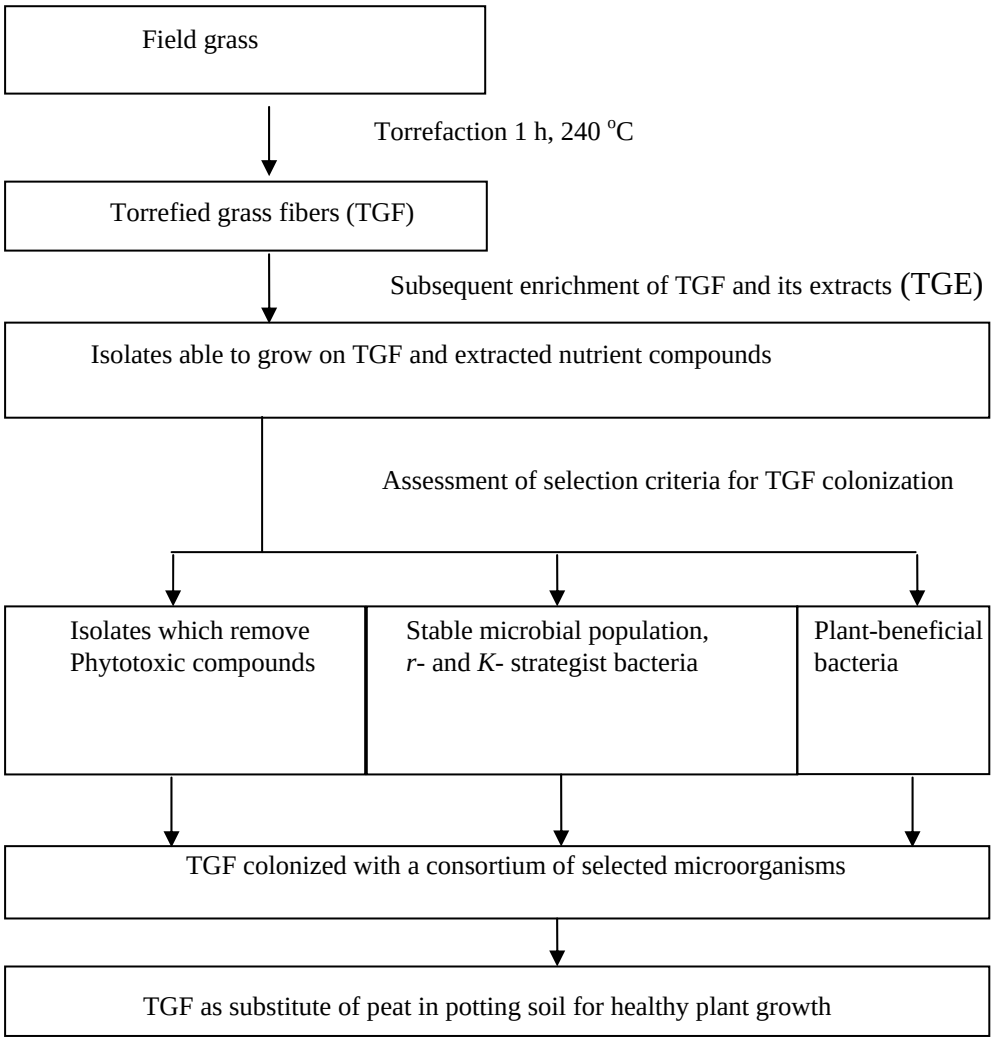

Fig. 2. Conceptual scheme of major steps in the work

Obviously, this research is driven by highly practical considerations, as it deals with the development of a novel substrate to be used in horticultural practice. Due to the lack of understanding of the microbial colonization of the novel substratum, different fundamental questions, as outlined above and below, had to be addressed.

Aims and research questions

Aims of the thesis

The general aims of this study were to assess:
1. what microorganisms will successfully colonize TGF and what drives such colonization,
2. can a microbial consortium be established that enhances the value of the TGF for use in potting mix.

We set out to isolate microorganisms from soil that would be part of a stable microbial consortium on the basis of presumed ecological behavior (growth) and function. Thus, a microbial consortium able to colonize TGF and persist on it, reduce the toxicity and have plant-beneficial properties was designed and tested for performance.

Research questions

In order to address the aims, six research questions were conceived:
1. What type of community introduced on TGF establishes and develops stably over time?

2. Can toxic compounds present in TGF be transformed by a biodegradative microflora?

3. What types of ecological interactions take place between fungal and bacterial parts of the consortium?

4. Will plant-beneficial (plant-growth-promoting or antagonistic) bacteria colonize TGF and stably persist on it?

5. Does microbially-colonized TGF constitute a suitable matrix for plant growth and can it be used as a substitute for peat?

Outline of the thesis

Chapter 1 introduces the scope of this thesis.
Chapter 2 discusses the type of microorganisms that are selected by TGF and TGF-extracted compounds. For that purpose, TGF and TGF extract were inoculated with a suspension of microorganisms obtained from soil. The microbial communities developing in the substrates during sequential microbial enrichment were assessed using cultivation-based and cultivation-independent approaches. Bacterial isolates were obtained and identified by partial sequencing of the 16S ribosomal RNA gene.

Chapter 3 focuses on the selection and analysis of isolates capable of removing particular toxic compounds from TGF and TGF extract. Eighty-eight bacterial strains and one fungus, isolated from sequential enrichment on TGF and TGF extract (chapter 2), were tested separately for their capacity to decrease phytotoxicity, using a lettuce seed germination assay. The organisms that significantly reduced toxicity were checked for their ability to grow on agar containing selected model compounds, i.e. 0.01% phenol, 2-methoxyphenol, 2,6-dimethoxyphenol, 2-furalaldehyde, pyrrole-2-carboxaldehyde and furan-2-methanol, as the sole C-sources. Gas chromatographic analyses of torrefied grass extracts determined which compounds were removed by the selected microorganisms.

In **chapter 4**, a consortium of bacteria suitable for beneficial colonization of TGF is designed. Selection criteria were based on growth in the enrichment experiment (chapter 2), removal of phytotoxicity (chapter 3), as well as on *in vitro* antagonism against plant pathogens. This designed microbial consortium was tested for its ability to colonize TGF. Unexpected problems were encountered with the levels of achievable bacterial colonization. Different combinations of bacteria, addition of particular nutrients and washing of the substrate – to remove putative water-soluble toxic compounds - were all tested for their efficacy to improve colonization. It was found that the presence of the ascomycete *Coniochaeta ligniaria* F/TGF15 was essential for establishment of the microbial consortium on TGF.

Chapter 5 assesses the interactions between the selected ascomycete *Coniochaeta ligniaria* F/TGF15 and selected gram-negative and gram-positive bacteria. Both the influence of these bacteria on the development of the fungus and the effect of the fungus on the growth of the bacteria were investigated. It was found that the fungus incited substantial bacterial movement and concomitant growth in the substrate.

In **chapter 6**, the quality of TGF as a new potting soil ingredient is tested in a greenhouse experiment. TGF was first colonized with different combinations of selected microorganisms, including *Coniochaeta ligniaria* F/TGF15, *Pseudomonas putida* 15/TGE5, *Serratia plymuthica* 23/TGE5, *Pseudomonas corrugata* 31/TGE5, *Methylobacterium radiotolerans* 56/TGF10, *Leifsonia xyli subsp. xyli* 66/TGF10, *Mycobacterium anthracenicum* 70/TGF15, and *Agromyces aurantiacus* 95/TGF15. Colonization under non-sterile conditions was assessed, as well as the effect on phytotoxicity. TGF was tested as a pure substratum or combined with peat for its quality as a potting soil ingredient. Tomato seedlings were used as test plants. It was proven that TGF could be a valuable component of potting soil.

CHAPTER 2

Thermally treated grass fibers as colonisable substrate for beneficial bacterial inoculum

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Abstract

This study investigates how thermally treated (i.e. torrefied) grass, a new prospective ingredient of potting soils, is colonized by microorganisms. Torrefied grass fibers (TGF) represent a specific colonisable niche, which is potentially useful to establish a beneficial microbial community that improves plant growth. TGF and torrefied grass extracts (TGE) were inoculated with a suspension of microorganisms obtained from soil. Sequential microbial enrichment steps were then performed in both substrates. The microbial communities developing in the substrates were assessed using cultivation-based and cultivation-independent approaches. Thus, bacterial isolates were obtained, and PCR-DGGE analyses for bacterial communities were performed. Partial sequencing of the 16S ribosomal RNA gene from isolates and bands from DGGE gels showed diverse communities after enrichment in TGE and TGF. Bacterial isolates affiliated with representatives of the α-proteobacteria (*Methylobacterium radiotolerans, Rhizobium radiobacter*), γ-proteobacteria (*Serratia plymuthica*, *Pseudomonas putida*), Cytophaga-Flavobacterium-Bacteroides (CFB) group (*Flavobacterium denitrificans*), β-proteobacteria (*Ralstonia campinensis*), actinobacteria (*Cellulomonas parahominis, Leifsonia poae, Leifsonia xyli subsp. xyli* and *Mycobacterium anthracenicum*) and the firmicutes (*Bacillus megaterium*) were found. In TGE, γ-proteobacteria were dominant (61.5% of the culturable community) and 20% belonged to the CFB group, whereas actinobacteria (67.4%) and α-proteobacteria (21.7%) were prevalent in TGF. A germination assay with lettuce seeds showed that the phytotoxicity of TGF and TGE decreased due to the microbial enrichment.

Introduction

Recently, increased attention has been paid to the recycling of solid organic waste for use as constituents in substrates for ornamental pot plant production [2, 27, 123]. Materials like hardwood, pine barks, composts from different sources and dried sewage sludge have been considered for this purpose [27, 42, 94, 96], and recently thermally treated grass has been suggested as a viable alternative .

Torrefaction is applied to protect grass fibers against decay and transform them into a stable product. The process relies on slow heating of grass biomass under anoxic conditions to a maximum temperature of 300^{o}C. The treatment yields a solid product with low moisture content and high energy content per unit mass. Torrefaction can be characterized as “mild pyrolysis”, with removal of gaseous compounds and formation of a solid end product. Approximately 70 % of the initial grass fiber weight and 80-90 % of the original energy content are retained [86]. Torrefaction leads to enhanced hydrophobicity and phytotoxicity [86] of the grass fibers. It is not clear yet which compounds from the torrefied grass fibers cause the phytotoxic effect. There are data that phenols and phenolic compounds cause phytotoxic problems [6, 91, 119].

As the torrefied grass fibers may represent a colonisable niche for microorganisms, it is important to know to which extent they can serve as colonization sites, providing niche space and nutrients such as bound carbon. Alterations in carbon availability will strongly affect microbial community structure and function, which has implications for the interactions with the plants to be grown in the substrate [48]. Multiple limiting nutrients [113], habitat heterogeneity [109], different colonization ability of microorganisms and other factors such as predation and microbial dormancy are responsible for the coexistence of microorganisms of different C-acquiring ability [48]. The types and availability of resources, in particular available carbon sources, in the TGF and TGE will thus determine the structure of the heterotrophic microbial community that establishes and the possible interactions within that community.

In this study, the colonization of TGE and TGF by soil microorganisms was assessed using a sequential enrichment procedure. Cultivation-based and DNA-based methods, i.e. PCR-DGGE, were applied to assess the microbial communities. A germination assay with lettuce seeds was carried out to analyze phytotoxicity of TGE and TGF.

Material and Methods

Origin of grass fibers and torrefaction process

Grass material was collected in August 2003 from unfertilized, semi-natural grassland on a peaty clay soil near Wageningen, The Netherlands. The biomass was harvested as a first cut and consisted of a mixture of more than 30 species (mainly grasses, rushes, sedges, and herbs) with a dry matter content of 37.5 %. Dry matter content of the plant biomass was measured by drying aliquots at 105^{o}C. The harvested material was dried at 105^{o}C, ground in a hammer mill to pieces <1 mm and stored until used for torrefaction experiments.

Samples of the ground material were torrefied in a preheated Carbolite muffle furnace (Model CWF 1100), at a temperature of 240^{o}C during 1 h. To ensure the occurrence of anoxic conditions, ground biomass was placed in aluminum boxes (approximately 40 g dry matter in a 200 ml box). The boxes were closed with their lid leaving a narrow split for gas exchange. The boxes, four at a time, were placed upside down in the oven to promote homogeneous heating of the contents. Boxes were weighed before and after heating to determine weight loss of the samples.

Torrefied and untorrefied grass was characterized chemically by the Energy research Centre of the Netherlands (ECN, Petten, and the Netherlands). Contents of C, H, N, and O were analyzed by Atomic Absorption Spectrometry (AAS) and mineral content (Ca, K, Mg, Na, P and S) was studied by Inductively Coupled Plasma - Mass Spectrometry

(ICP-MS) (Table 1). Table 2 shows the compounds which were detected by gas chromatography-flame ionization (FI) using a gas chromatograph with a ZB-WAX column (30 m x 0.25 mm df = 0.25 µm). The carrier gas was He. The oven was programmed at an initial temperature of 40°C for 5 min with a ramp of 5°C/min to 245°C. Formate was measured by ECN using ion chromatography. Table 2 summarizes the compounds measured in untreated and torrefied grass. Several compounds that were suggested to play a role in phytotoxicity, were not found, they were below the detection limit of the applied techniques. These compounds are given in the footnote of the table.

Table 1. Chemical composition of untreated and torrefied (240 ^{0}C, 1 h) plant biomass.

	C	H	N	O	Na	K	Ca	Mg	P	S
	% of Dry matter									
Untreated	46.3	5.85	1.17	44.1	0.36	0.55	0.73	0.24	0.10	0.21
Torrefied	51.6	5.45	1.35	35.4	0.42	0.66	0.88	0.30	0.12	0.24

Grass fiber extraction

Extracts of the grass fiber matrix were prepared by the following protocol: 9.5 g of torrefied grass fiber was cold-extracted at room temperature with 250ml distilled water for 2 h using a magnetic stirrer at 700 rpm. Following the cold extraction, the suspension was paper-filtered to remove grass fiber particles. The pH was adjusted to 6.8. The TGE was then filter-sterilized (Millex GV bacterial filter Unit 0.22 µm Millipore) and stored at 4 ^{0}C.

Microbial inoculum from soil

To provide a fresh inoculum for inoculation of TGE and TGF, a sandy soil sample was collected from a fire place exposed some times per year for at least 20 years to high temperature (Wageningen, the Netherlands); this soil was denoted “Fireside” soil. It was chosen after a preliminary experiment where two other types of soil, arable soil and forest soil, were used to enrich TGF. However these enrichments insufficiently removed the phytotoxicity as tested by lettuce seed germination.

A soil suspension from Fireside soil was prepared by mixing 10 g of soil with 90 ml 0.1 % tetra sodium pyrophosphate (NaPP- $Na_2P_2O_7.12H_2O$, Merck), and 10 g gravel (2 mm dia) in 250 ml Erlenmeyer flasks. The flasks were shaken for 30 min at 250 rpm.

Sequential microbial enrichment in TGE

Ten ml sterile TGE (pH 6.8) were mixed with 10 ml sterile minimal salts medium (MSM).

Table 2.Compounds detected by gas chromatography (GC) in untreated and torrefied plant biomass.

Compound	Untreated grass mg/kg dry material	Torrefied grass mg/kg dry material
Formate	<20	791
Acetate	185.5	465.6
Hydroxyaceton	12	29.4
2-Furaldehyde	<5	27.9
1-hydroxy-2-butanon	<5	14.7
Phenol	9.8	11.2
2-Methoxyphenol	3.9	10.8
2,6- Dimethoxyphenol	<10	10.2
Furan-2-methanol	<5	8.6
Pyrrole-2-carboxaldehyde	<5	6.8
Cyclohexanon	<2	3.5

Compounds below the detection limit which is indicated between brackets are: propionic acid (<10 mg/kg) 1,2,4-trimethoxybenzene (<5 mg/kg), 2(5H)-furanon (<10 mg/kg), 2-butenal (<2 mg/kg), 3-methoxypyridine (<10 mg/kg), 3,4,5-trimethoxytoluene (<5 mg/kg), 5-(hydroxymethyl) -2-furaldehyd (<10 mg/kg), 5-methyl-2-furaldehyde (<5 mg/kg), acetaldehyde (<50 mg/kg), acetone (<2 mg/kg), etanol (<5 mg/kg), isoetanol (<10 mg/kg), methanol (<50 mg/kg), methylacetate (<5 mg/kg), methylformate (<2 mg/kg), propanal (<2 mg/kg)

MSM ingredients were K_2HPO_4 1.4 g/L, KH_2PO_4 1.4 g/L, $MgSO_4.7H_2O$ 0.1 g/L, $(NH_4)_2SO_4$ 1 g/L. Then, 0.2 ml of Fireside soil suspension was added to the TGE/MSM mixture. The flasks were incubated for 5 d at 180 rpm, 25^{o}C in a Gallenkamp orbital shaker. After 5 d, an aliquot of 0.2 ml from the first inoculated flask (denoted N1) was transferred to flask N2, containing 20 ml fresh growth medium TGE:MSM (1:1). Thus, a 100-fold dilution was achieved. The flasks were incubated as described above. This procedure was performed 20 times in two parallel series. At set times samples were taken for analyses of the microbial communities for PCR-DGGE, CFU and phytotoxicity reduction.

Sequential microbial enrichment in TGF

The enrichment in TGF was based on an inoculum prepared in 500 ml Erlenmeyer flask containing 10 g sterile gravel, 10 g Fireside soil and 90 ml MSM medium. The culture was shaken for 30 min at 250 rpm in an orbital incubator (Gallenkamp).

Subsequently, 1.6 ml from the soil suspension was added to 0.5 g of γ-sterilized (25 kGray) TGF in 50 ml vessels, establishing 60 % of the substrate's water holding capacity. The systems were incubated for 7 days at 25^{o}C. Following incubation, 5 ml of fresh MSM was added to the 7 d incubated vessels, and mixed well. A fresh inoculum of 1.6 ml was transferred from this vessel into new vessels containing 0.5 g sterile TGF, and these novel TGF cultures were again incubated (7 d, at 25^{o}C). The experiment was carried out in two series, and the transfer procedure was repeated 15 times. Samples were taken and analyzed for PCR-DGGE and CFU and phytotoxicity reduction.

Phytotoxicity assay

A lettuce (*Lactica sativa,* cultivar Erika, Enza Zaden, Enkhuizen, the Netherlands) seed germination test was designed to monitor the phytotoxicity of the TGE. Monitoring of enrichment steps 1, 5, 10, 15 and 20 in TGE was done by using 24-well plates (Greiner Bio-one, CELLSTAR®, Germany). Bacterial cells were removed from the TGE enrichments by centrifugation (9.400 x *g*, 10 min) and the supernatants were filter-sterilized (Millex GV bacterial filter Unit 0.22 µm, Millipore). Then, 250 µl from the resulting filtrate was used per well. The test was performed in six wells in a randomized design, each with 5 seeds. After 48 h, the number of seeds that had germinated was counted. As controls, distilled H_2O and TGE: MSM=1:1 were used.

The seed germination test performed with microbial-enriched TGF was slightly modified from the one in TGE. After 7 d of microbial growth on TGF, 13.2 ml distilled water were added to the fibers and extraction at room temperature was performed for 2 h using a magnetic stirrer at 700 rpm. The solution was paper-filtered to remove the fibers. Bacterial cells were removed by centrifugation (9.400 x g, 10 min), followed by filter-sterilization as described above. Three treatments were compared: TGF without inoculum (TGF - MO), TGF plus bacterial inoculum transfer 5 (TGF5) and TGF plus bacterial inoculum transfer 15 (TGF15). The samples were kept undiluted and diluted 1:4 and 1:16 in distilled water.

Bacterial plate counts

Colony-forming units (CFU) were counted after enrichment steps 3, 5, 10, 15 and 20 in TGE and after enrichment steps 1, 3, 5, 10 and 15 of the sequential enrichment experiment in TGF. Ten-fold dilution series were prepared in 0.85% NaCl and plated on R2A medium (Difco, Detroit, MI, USA) supplemented with delvocit (100 mg/L) to inhibit

fungal growth. Results are presented as logarithmic numbers of CFU per ml TGE and per g (oven-dry) TGF.

DNA extraction from TGE and TGF

Total community DNA from enrichment steps 1, 2, 3, 5, 10, 15 and 20 (TGE) and 1, 2, 3, 5, 10 and 15 (TGF) was isolated and purified with the UltraClean™ soil DNA kit (MoBio Laboratories, BIOzymTC, Landgraaf, the Netherlands) according to the protocol of the supplier, except that the cells were disrupted by bead beating 4 times (30 s each time) in a Braun's cell homogenizer (Braun, Melsungen, Germany) at maximum speed. For disruption, glass beads (50 mg, 0.11 mm dia) were added to 2 ml of TGE and 0.25 g of TGF respectively. The bead beating step was included to ensure maximal cell lyses without severe shearing of the DNA. DNA quality and quantity were assessed by electrophoresis in 0.8 % agarose gels in 0.5 x TBE buffer [103] by comparison to a standard 1-kb ladder (Invitrogen, Cat.15615-024, Carlsbad, USA). DNA size was 10-40 kb, on average.

PCR amplification

Amplification of 16S ribosomal (r) RNA genes was performed by using PCR in 50-µl reaction volumes containing 0.2 µM of each primer, 3.75 mM $MgCl_2$ (Perkin-Elmer, Nieuwersluis, the Netherlands), 200 µM of each dNTP (Boehringer, Almere, the Netherlands) and 0.25 µg of T4 gene 32 protein (Boehringer, Mannheim, Germany) using 5 U Ampli*Taq* Stoffel fragment in Stoffel buffer. The primers used were bacterial 16S rRNA gene forward primer U968 (5'-AACGCGAAGAACCTTAC-3') and reverse primer R1378 (5'-CGGTGTGTACAAGGCCCGGGAACG-3') [62]. For DGGE, a GC-clamped version of U968, i.e. U968-GC (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAAC CTTAC-3') was used. Thermal cycling was as follows: initial denaturation at 94^0 C for 5 min, followed by 35 cycles of 94^oC for 1 min, 65^oC for 90 s, 72^oC for 2 min, final extension at 72^oC for 10 min. The PCR products (expected sizes about 450 bp) were analyzed by running 5 µl aliquots of the reaction mixtures in 1 % agarose gels.

DGGE analyses

DGGE analyses [61] were performed using 6% polyacrylamide gels (ratio of acrylamide to bisacrylamide 37:1) with a gradient of 45 % to 65 % denaturants (100 % denaturant was defined as 7 M urea plus 40% formamide). The gels were run at 60 ^{0}C (100 V) for 16 h in a Phor-U2 apparatus (Ingeny International, Goes, the Netherlands) and stained with SYBR gold (Molecular Probes, Leiden, the Netherlands). For analysis of the molecular community profiles, the Molecular Analyst fingerprint software (version 6.0, Biorad, Veenendaal, the Netherlands) was used.

Recovery, purification and sequencing of DNA template from DGGE bands

Organisms underlying the DGGE profiles were analyzed by reamplifying and sequencing bands of interest. DNA was eluted from excised bands as described by Salles [101]. Crushed excised bands were incubated in 20 µl of sterile H_2O at 65^{o}C for 30 min, followed by centrifugation at 5000 x *g* for 1 min. The eluted DNA was used as the template for PCR amplification. Selected bands were reamplified using the clamped PCR system and run again on DGGE gel. Only bands with migratory positions identical to those originally found were used for subsequent DNA sequence analysis.

Purified PCR products were subjected to a final cleaning step using the High-pure PCR purification kit (Roche GmbH, Mannheim, Germany) and then sequenced using an ABI Prism automatic sequencer (Greenomics, Wageningen, The Netherlands). The partial 16S rRNA gene sequences were compared with those available in the NCBI database using BLAST-N. The sequences generated were deposited in the GeneBank under the accession numbers: EU287845 to EU287853.

Identification and sequencing of bacterial strains

In total 111 bacterial strains were isolated and maintained as pure cultures on R2A medium at 20^{o}C. Long-term storage was in glycerol stocks at -80^{o}C Crude DNA was obtained from all 111 strains as described by Salles [100]. Briefly, one freshly-grown colony per strain was resuspended in 20 µl of sterile Milli-Q water in a microfuge tube. Cells were lysed by keeping the suspension at 95^{o}C for 10 min, followed by cooling on ice for 10 min. Before performing lyses, 5 mg of Chelex-100 resin (Sigma-Aldrich BV, Zwijndrecht, the Netherlands) was added to the microtubes. By this procedure, inhibitory compounds released from lysing cells were sequestered by the resin, and subsequent centrifugation (13.400 x *g*, 5 min) removed them from the suspension. The microfuge tubes containing the crude lysates were stored at -20^{o}C.

The crude lysates of the strains were used as a template for PCR reaction for sequencing. This was done as it was described in section PCR amplification. The PCR products were purified with High-pure PCR purification kit (Roche GmbH Mannheim, Germany) and then sequenced using an ABI Prism automatic sequencer (Greenomics, Wageningen, the Netherlands).

The partial 16S rRNA gene sequences were investigated by DNAstar v6/Seqman program and compared with those available in the NCBI database using BLAST-N. The sequences generated were deposited in the GeneBank under the accession numbers: EU293366 to EU293390. Since many of the strains had a repeatable hit in the NCBI database (BLAST-N), only one representative sequence of each group of strains was send to the GeneBank.

Carbon substrate utilization test

The 111 strains isolated from the TGE and TGF enrichment experiment were grouped in 17 groups by BOX-PCR (data not shown). Representative strains from each group were tested in duplicate for growth on the main readily-available carbon sources in TGF, acetic acid and potassium formate. Bacterial colonies grown on R2A were inoculated into Erlenmeyer flasks (50 ml) containing MSM, as described previously, supplemented with either acetic (1 %) or formic acid (1 %) and 0.001 % yeast extract, or a combination of both carbon sources, i.e. 0.5 % acetic acid and 0.5 % K-formate, and 0.001 % yeast extract. After adjustment of the pH to 6.9, the solutions were filter-sterilized (Millex GV bacterial filter Unit 0.22 µm Millipore) before bacterial inocula were added. The Erlenmeyer flasks were shaken at 25°C (180 rpm). Bacterial growth was recorded by increase of OD_{600}, using a BECKMAN DU® 530 Life Science UV/Vis spectrophotometer at regular time intervals, i.e. on days 2, 4, 6, 8 and 10. OD_{600} >0.1 was regarded as positive.

Statistical analyses

Analyses of variance (ANOVA) were performed for CFU enumerations and germination data with the statistical program Genstat 8 (Rothamsted Experimental Station, Harpenden, UK). Least significant differences (LSD) were calculated at a significant level of P=0.05. The CFU data were expressed as mean values over triplicate experimental units and transformed to logarithmic values. The germination percentages were analyzed by two-way ANOVA: the significance of the enrichment step and replicate series were analyzed in TGE, and the significance of the enrichment step and the dilution were analyzed in TGF.

Cluster analyses of DGGE patterns were performed by GelCompar II (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Dendrograms were generated based on UPGMA and Pearson correlation that represented the similarities between the PCR-DGGE profiles.

Results

Bacterial plate counts

The bacterial numbers increased from log 5.2 (starting inoculum) up to log 8.2 (after the first enrichment step) and showed a successive increase to log 9.2 per ml in the last enrichment steps (15 and 20), (Figure 1). There was no significant difference between replicate series.

The number of bacteria initially introduced onto the TGF was log 8.4 CFU/g (Figure 2). After the first transfer, the CFU number reached log 9.1 per g. CFU numbers

increased during the ensuing enrichment stages up to log 10.2 per g in transfer 15. There was no significant difference between replicate series.

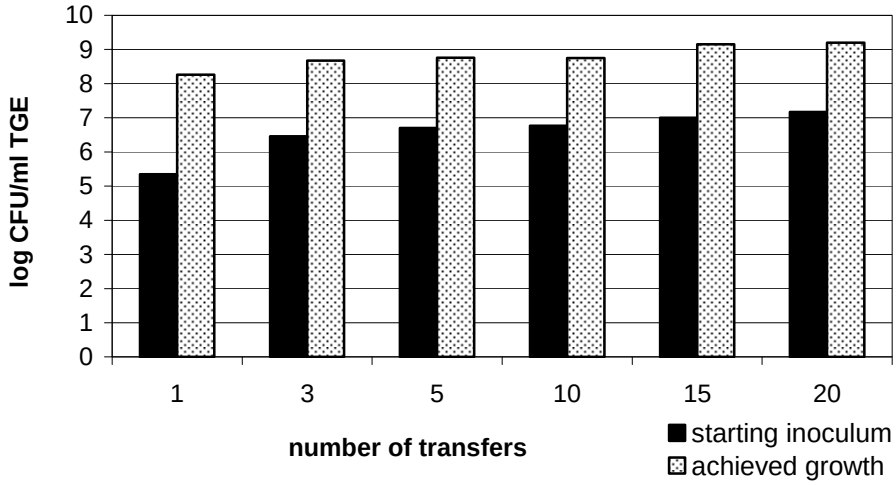

Fig. 1 Bacterial numbers (log CFU/ml) of starting inoculum and achieved growth after 5 days of colonization in sequential enrichment steps in TGE.
Least significant difference is 1.43 (P=0.05).

Phytotoxicity assay

Lettuce seed germination in TGE was significantly enhanced by the microbial enrichment. TGE without microbial colonization (untreated TGE:MSM=1:1) only yielded 3 % germination, whereas the percentage of germinated seeds varied between 17 % and 62 % in the enriched TGE (Figure 3). Germination was improved significantly in TGE1, TGE5, TGE15 and TGE20 compared to the uninoculated control. The two replicate series were not significantly different (P>0.05). In the water control, all seeds germinated.

No seeds germinated in the treatments TGF without MO and TGF5. In contrast to these results, TGF15 showed 26.7 % germination (Figure 4). Dilutions of 1:4 and 1:16 were included for all three treatments to obtain better contrasts in the phytotoxic assay of TGF. Using 1:4 and 1:16 dilution for TGF5, the germination was increased up to 76.7 % and 100 % respectively, whereas seed germination in TGF without MO was only 63 % and 75 %

respectively. TGF15 gives always significantly higher percentage of germination compared to uncolonized TGF.

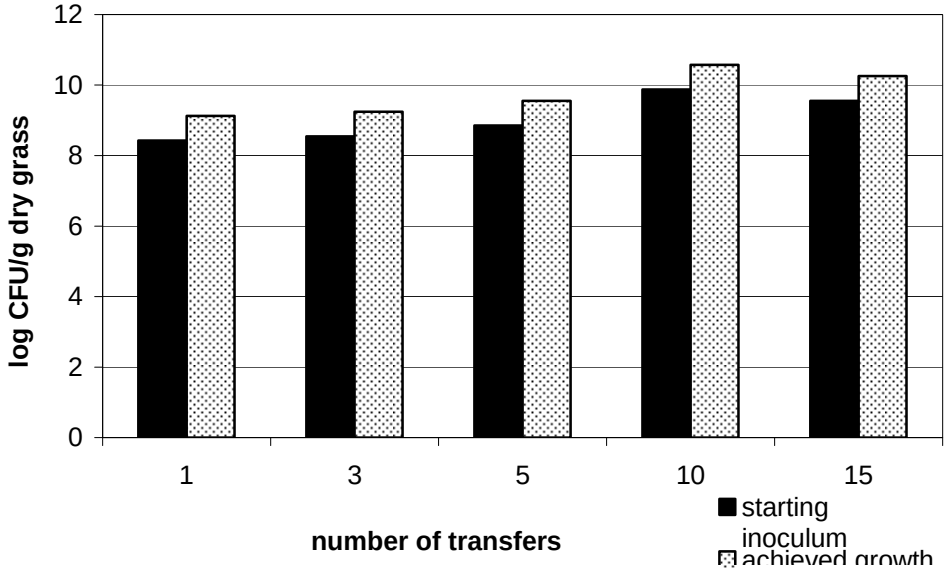

Fig. 2 Bacterial numbers (log CFU/g) of starting inoculum and achieved growth after 7 days of colonization in sequential enrichment steps in TGF. Least significant difference is 0.32 (*P*=0.05).

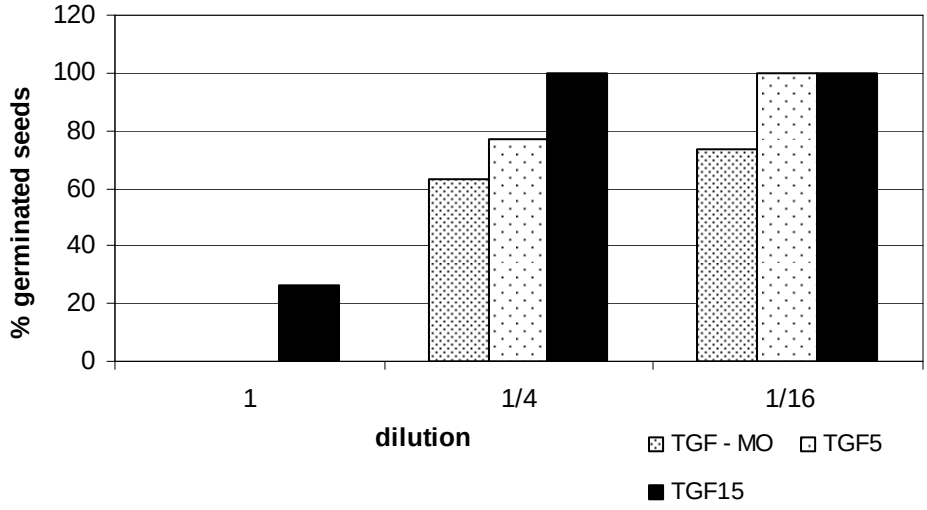

Fig 3 Lettuce seed germination after sequential microbial enrichment steps in TGE, compared to germination in a water control and TGE without microorganisms (TGE-MO).Least significant difference is 18.2 (*P*=0.05).

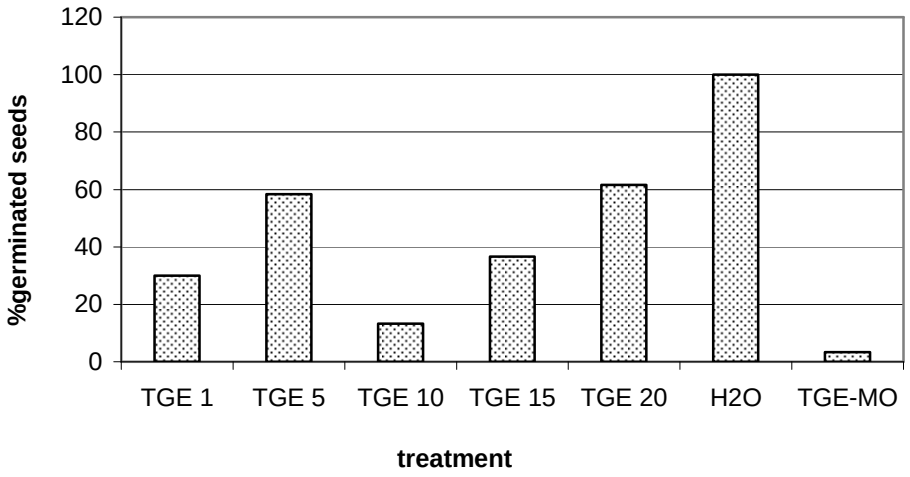

Fig. 4 Lettuce seed germination after sequential microbial enrichment steps in TGF, compared to germination in TGF without microorganisms (TGE-MO). The treatments were tested undiluted (1) and diluted 4 and 16 times.
Least significant difference is 18.9 (*P*=0.05).

DGGE fingerprints of TGE and TGF

The bacterial community colonizing TGE showed different and shifting levels of complexity during the subsequent enrichment steps (Figure 5A). In TGE1, the banding patterns were complex and multiple showing at least 17 bands. At increasing enrichment steps, fewer bands occurred and these became more pronounced. The banding patterns in both replicates of every enrichment step, except for 1a and 1b, had virtually the same number of bands and position, which confirms the consistency between replicates. In TGE2, TGE3 and TGE5 approximately 14 bands were present. Thereafter, in TGE10, TGE15 and TGE20 the number of bands had decreased and a balanced community with about 10 bands had developed.

Few bands (Figure 5A, bands 1, 2 and 4) dominated in the patterns between the TGE2 and TGE20 enrichment step. This may indicate the selection of a specific bacterial type as a result of substrate or ambient adaptation.

A dendrogram was constructed based on band composition (Figure 5B). All replicates (except in 1a, 1b and 3a, 3b) were clustered together, showing relatively low inter-replicate variability. Two large clusters were found, with similarity levels between them of 60 %. One cluster included all samples from 10 through to 20, whereas the other one encompassed all samples 2 to 5. Samples 1a and 1b were quite different from all the other samples. Within the two main clusters, smaller sub-clusters, in accordance with transfer number (both higher than 70 % similarity) were observed.

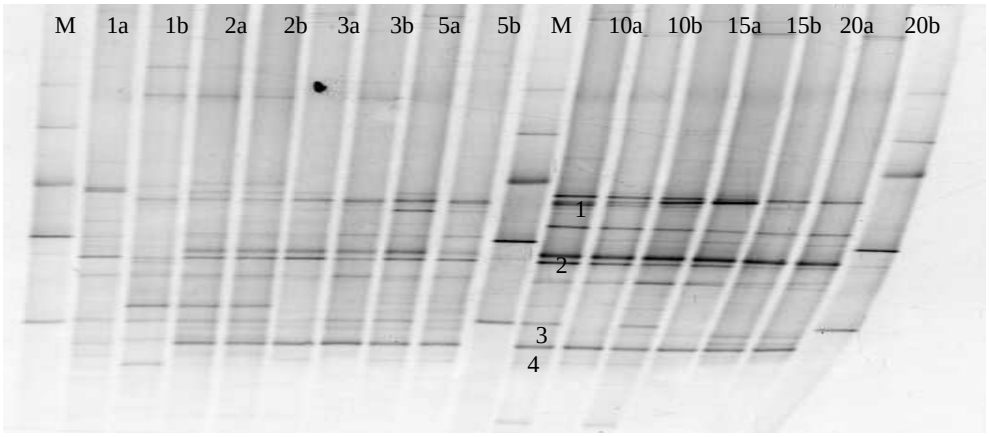

Fig.5a PCR-DGGE banding patterns of torrefied grass extract at different sequential enrichment steps in duplicate series a and b. M is a bacterial marker. Numbers in the gel represent sequenced bands.

Figure 6A shows the DGGE bacterial fingerprints obtained from the TGF samples. The bacterial communities apparently reached stability after five transfers, as it can be seen from the convergent profiles obtained in the enrichment steps 5, 10 and 15. Enrichment step 5 thus seems crucial because the main bacterial selection occurred around this phase. There were few very strong bands (Figure 6A, bands 5, and 6), which appeared as from the third enrichment step and were present until the end of the experiment. Consistency of number and location of bands between the two replicates was observed during every enrichment step. After enrichment step 1, the profiles consisted of many bands, occurring close to each other. This created difficulties to count their exact number. After enrichment step 2, about 20 bands were counted for both replicates, while after enrichment step 3 the number of bands was reduced to up to 14 with a slight difference in band numbers between the two replicates. After enrichment step 5, a clear banding pattern consisting of 12 bands emerged. This pattern remained consistent throughout the next enrichment steps, i.e. 10 and 15. One

band (Figure 6A, band 7), which was present in enrichment steps 1, 2, 3 and 5, disappeared in enrichment steps 10 and 15.

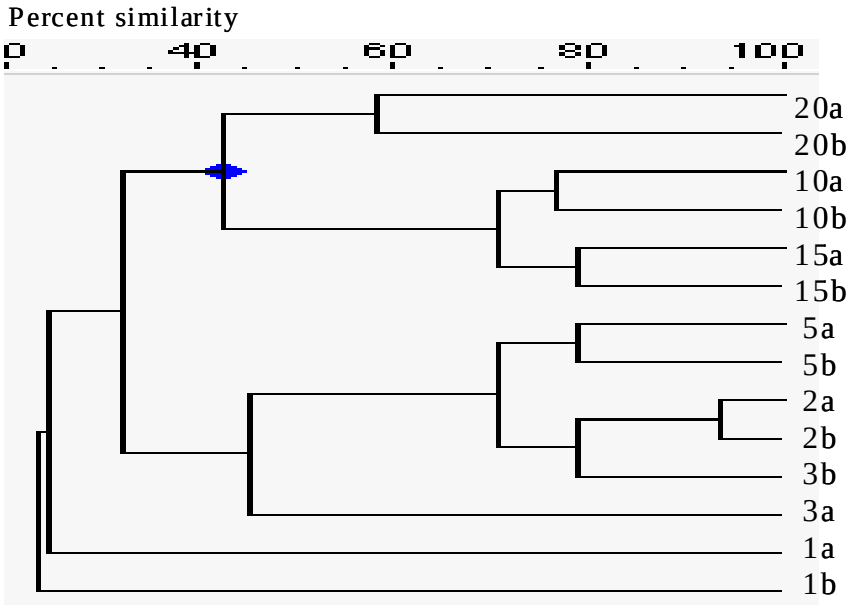

Figure 5b A dendrogram representing the similarity of PCR-DGGE profiles generated with bacterial DNA from different enrichment steps in TGE

According to the dendrogram (Figure 6B), two clear clusters were formed, enrichment 1, 2 and 3 with similarity of the profiles of about 94 % and enrichment 5, 10 and 15 with 96 % similarity. The main clusters were each divided in sub-clusters which tended to group replicates together.

Identification of bands in PCR-DGGE patterns from TGE and TGF

Selected bands present in TGE (Figure 5A, Table 3 bands 1, 2, 3, and 4) and TGF (Figure 6A, Table 3 bands 5, 6, 7, and 8) fingerprints were excised, reamplified and sequenced. A database analysis revealed that the closest matches to the bands in TGE, encompassed a bacterium belonging to the Cytophaga-Flavobacterium-Bacteroides (CFB), i.e. *Flavobacterium denitrificans* (97.7 %, band 1), and three bacteria belonging to γ-proteobacteria, i.e. *Stenotrophomonas maltophilia (*95.5 % band 2*) Pseudomonas gingeri* (98.7 % band 3), *Pseudomonas putida (*98.7 % band 4).

In the TGF system, we found evidence for the occurrence of strains affiliated with *Afipia broomeae*, (98 %, band 5, α-proteobacteria) and *Arthrobacter chlorophenolicus*,

(97.6 %, band 6, actinobacteria), *Agromyces aurantiacus (95* %, band 7), and *Streptomyces paresii* (99.1 %, band 8).

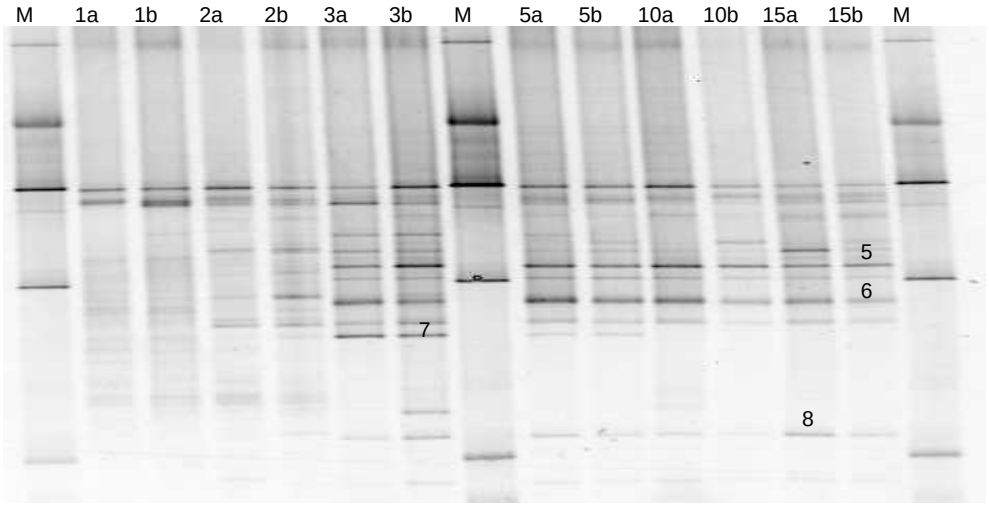

Fig.6a PCR-DGGE banding pattern of torrefied grass fiber (TGF) after different sequential enrichment steps in duplicate series a and b; M is a bacterial marker.

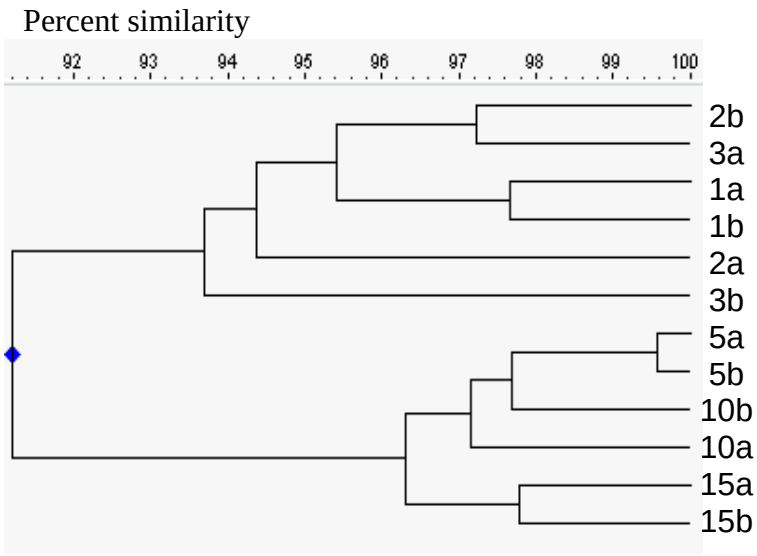

Figure 6b is a dendrogram representing the similarity of PCR-DGGE profiles generated with bacterial DNA from different enrichment steps in TGF.

Identification of bacterial isolates

In total, 111 bacterial strains were isolated from the sequential enrichments, respectively 37, 28, 12, and 34 from TGE5, TGE20, TGF10, and TGF15. The sequencing results show the most abundant strain in TGE5 was affiliated to *Pseudomonas plecoglossicida,* while *Flavobacterium denitrificans* dominated in TGE20 (Table 4).

The bacterial communities in TGF10 and TGF15 consisted mainly of Gram-positive bacteria (Table 4). The most frequently found strain in TGF15 was identified as *Leifsonia poae.* Other less dominating strains in TGF15 were *Mycobacterium anthracenicum* and *Leifsonia xyli subsp. xyli* In TGF10, *Bacillus megaterium, Methylobacterium radiotolerans and Leifsonia xyli subsp. xyli* shared almost the same number of strains (3, 3 and 4 respectively). *Cellulomonas parahominis* and *Tsukamurella poriferae* were less frequently found. The partial 16S rRNA analysis of the isolates from the different enrichment steps on TGE and TGF showed bacterial communities of limited diversity. Bacteria adapted to TGE were mainly Gram-negative in a contrast with the ones isolated from TGF, which were mainly Gram-positive.

Taking both TGE5 and TGE20 into account, 61.5% belonged to γ-proteobacteria, 20 % to the Cytophaga-Flavobacterium-Bacteroides, 6.2 % to β-proteobacteria, 9.2 % to the actinobacteria and 3.1% to the α-proteobacteria. In contrast, isolates from TGF10 and TGF15 belonged for 67.4% to actinobacteria, 6.5 % firmicutes, 21.7 % α-proteobacteria and only 4.3% were γ-proteobacteria.

In the TGF system, we found evidence for the occurrence of strains affiliated with *Afipia broomeae,* (98 %, band 5, α-proteobacteria) and *Arthrobacter chlorophenolicus*, (97.6 %, band 6, actinobacteria), *Agromyces aurantiacus (95* %, band 7), and *Streptomyces paresii* (99.1 %, band 8).

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diversity. Bacteria adapted to TGE were mainly Gram-negative in a contrast with the ones isolated from TGF, which were mainly Gram-positive. Taking both TGE5 and TGE20 into account, 61.5% belonged to γ-proteobacteria, 20 % to the Cytophaga-Flavobacterium-Bacteroides, 6.2 % to β-proteobacteria, 9.2 % to the actinobacteria and 3.1% to the α-proteobacteria. In contrast, isolates from TGF10 and TGF15 belonged for 67.4% to actinobacteria, 6.5 % firmicutes, 21.7 % α-proteobacteria and only 4.3% were γ-proteobacteria.

Comparing bands identified in the TGE and TGF systems (Table 3) with the identified strains in TGE and TGF (Table 4), there was a clear match between most of the identified bands with some of the isolated bacteria. For example, *Flavobacterium denitrificans, Stenotrophomonas maltophilia* and *Pseudomonas putida* were found as bands in TGE derived from DGGE profiles and also as isolates from TGE. A similar link was observed for *Afipia broomeae* and *Agromyces aurantiacus.* Those species were found as bands and also as isolates from TGF. However, *Streptomyces* was detected in the DGGE banding pattern of TGF, but was not present among the isolated bacteria.

Carbon substrate utilization

Out of the 17 strains tested, only four strains, denoted 4, 23, 69 and 70, grew on MSM medium supplemented with acetic acid as sole carbon source (plus 0.001 % yeast extract), whereas only two strains (56 and 70) grew on MSM medium supplemented with 1 % K-formate (plus 0.001 % yeast extract). In contrast, 16 out of 17 strains grew well on MSM medium supplemented with 0.5 % acetic acid and 0.5 % K-formate, in the presence of 0.001% yeast extract (Table 5). The bacteria in the yeast extract itself did not spur growth.

Discussion

In this study, TGE and TGF were inoculated with a microbial inoculum obtained from soil in order to assess which soil microorganisms can stably colonize these systems. For that purpose, sequential microbial enrichments were performed. Using the enrichments, we aimed to select those microorganisms that might be adapted to the matrix and/or its carbonaceous compounds. After 20 sequential steps of enrichment in TGE and 15 in TGF, which was estimated to be equivalent to 140 and 43 generations respectively, presumably microorganisms adapted to the prevailing conditions in the two systems were selected, and knowledge was obtained regarding the community compositions. Table 2 shows 11 compounds that were present after the torrefaction. Some of these compounds may serve as easily available carbon source for many bacteria. Two of these occurred in amounts that could potentially spur considerable microbial growth, i.e. acetate (465.6 mg/kg) and

Table 3. Closest matches for 16S rRNA gene sequences of bands excised from PCR-DGGE banding patterns of TGF and TGE

Band origin	Closest relative in GenBank	Percentage similarity	GenBank accession No	Reference	Closest type strain	Percentage similarity	Type strain GenBank accession No	Reference
1 TGE	*Flavobacterium denitrificans* .	97.7	AJ318907	(12)	*Flavobacterium denitrificans*	94.6	AJ704214	(9)
2 TGE	*Stenotrophomonas maltophilia;*	95.5	EU263112 AM114434	-	*Stenotrophomonas maltophilia*	94.7	AB008509	(13)
	Uncultured alpha proteobacterium	96		(5)				
3 TGE	*Pseudomonas gingeri*	98.7	AF320991	(7)	*Pseudomonas pictorum*	94.7	AB021392	(2)
4 TGE	*Pseudomonas putida/Pseudomonas plecoglossicida*	98.7/98.9	DQ229315/ AJ245436	-/(14)	*Pseudomonas plecoglossicida*	98.7	AB009457	(23)
5 TGF	*Afipia broomeae*	98	AY568506	-	*Afipia massiliensis*	98	AY029562	(17)
6 TGF	*Arthrobacter chlorophenolicus*	97.6	AF102267	(40)	*Arthrobacter chlorophenolicus*	97.6	AF102267	(40)
7 TGF	*Agromyces aurantiacus*	95	AF389342	(4)	*Agromyces aurantiacus*	95	AF389342	(19)
8 TGF	*Streptomyces paresii*	99.1	AJ969177	-	*Streptomyces atroolivaceus*	98.5	AJ781320	(18)

formate (791 mg/kg). As we assumed, the selected strains could grow on minimal media supplied with acetate and formate as sole carbon sources. Sixteen out of seventeen isolates grew on the combination of both carbon sources. However, only few strains utilized formate and acetate separately. Some of the other compounds, namely 2-furaldehyde (27.9 mg/kg), phenol (11.2 mg/kg), 2-methoxyphenol (10.8 mg/kg), 2,6-dimethoxyphenol (10.2 mg/kg) and furan-2-methanol (8.6 mg/kg) might be involved in phytotoxicity, but could also serve as a nutrient source.

The CFU on TGF were high (10^9 -10^{10} /g) in comparison with other organic substrates such as potting soil or compost which contain 10^8 - 5 x 10^9 bacterial CFU [90]. The bacterial numbers in TGF always increased from a diluted inoculum to more or less the same high level. This is an indication that torrefied grass fibers have a large carrying capacity. In other words, they offer specific colonisable niches for microorganisms and provide substrates and space for microbial development. In this sense, fiber's carrying capacity is the amount of microorganisms that could live on the torrefied grass fibers. Consequently, the fibers are expected to be a good substrate for microorganisms. The clear reduction of number of bands on DGGE during the enrichment steps, in both systems TGE and TGF indicated that the bacterial community was increasingly adapted to the TGE and TGF environment over transfer steps. Moreover, all DGGE fingerprints obtained from TGE and TGF over time showed considerable differences between the two systems. This was understandable in ecological terms, as the physical nature of the substrate was different, i.e. a liquid, well-aerated system in the case of TGE versus a solid-dominated and possibly locally oxygen-limited system in the case of TGF. Hence, in our selection procedure we mimicked two sets of widely divergent ecological conditions, which in turn were selective for two divergent microbial community structures. Sequencing analyses of bands from TGE and TGF revealed phylogenetic differences. Cytophaga-Flavobacterium-Bacteroides (CFB) and γ-proteobacteria were typical for TGE, and α-proteobacteria and actinobacteria were found in TGF. The differences in microbial groups might be also related to the differences in the physical nature of both substrates.

Intriguingly, consistent and stable patterns of number and position of bands were achieved after few enrichment steps in both systems. The communities in TGE and TGF ended up with about 10 bands, an indication that the community contains several bacteria living together on the same substrate. Few bands (Figure 5A) found in the fingerprint of the first sequential enrichment, disappeared in later enrichment steps, possibly because of poor adaptation of the underlying organism to the applied conditions.

Comparing the bands from TGE and TGF DGGE fingerprints in the final enrichment steps with the sequences of the isolates obtained from both systems, almost all bands from the TGE and some from the TGF had a corresponding strain.

Table 4. Closest relatives of the isolates from TGE and TGF

The closest related bacterial sequence	Accession No	% identity	Numbers of strains isolated from TGE5	Numbers of strains isolated from TGE20
Arthrobacter nicotinovorans	AY833102	99.5	6	-
Flavobacterium denitrificans	AJ318907	99.2 - 91.7	3	10
Pseudomonas corrugata	EF153018	99.7	1	-
Pseudomonas putida	DQ229317	99.2	3	6
Pseudomonas plecoglossicida	DQ095892	96	17	7
Ralstonia campinensis	AF312020	97.6	1	3
Rhizobium radiobacter	AY626383	98.6	-	2
Serratia plymuthica	AY 551332	100	2	-
Stenotrophomonas maltophilia	AY841799	99.5 - 97.5	4	-
			Numbers of strains isolated from TGF10	Numbers of strains isolated from TGF15
Agrococcus casei	DQ168427	98.7	-	1
Agromyces aurantiacus	AF389342	97.2	-	1
Afipia broomeae	U87760	97.1	-	2
Bacillus megaterium	DQ207561	99.8	3	-
Cellulomonas parahominis	AY655729	99.7	1	3
Leifsonia poae	AM410682	97.7	-	16
Leifsonia xyli subsp. xyli	DQ232616	98.7	4	4
Mycobacterium anthracenicum	MSY15709	98.4	-	7
Methylobacterium radiotolerans	AY616142	99.5-94.4	3	-
Tsukamurella poriferae	AY714240	100	1	-

However, *Streptomyces* was not present among the isolated bacteria.

Interestingly, several sequences of bands as well as isolates corresponded with sequences from isolates originating from rather extreme, sometimes toxic environments. For example, the sequence determined for band 2 identified as remotely related to *Stenotrophomonas maltophilia* was discovered in uranium mining waste, in which heavy metals and radionuclides abound [32]. In total 9 isolates from TGE5 and TGE20 were affiliated with *P. putida* (99.2 %) which, according to NCBI Database was shown to be tolerant to (and possibly consuming) toluene. Isolates from TGF10 were identified as akin to *Bacillus megaterium* (99.8 %) and this strain was obtained from "naphtha" water transporting pipelines [93]. Interestingly, band 6 was identified as *Arthrobacter chlorophenolicus*, a species capable of degrading high concentrations of 4-chlorophenol [81]. Band 5 affiliated with *A. broomeae* which was similar to a sequence found in radioactive waste [77]. *Flavobacterium* is known as a bacterium utilizing furfural [70]. *Methylobacterium* possess rather diverse characteristics from producing 1-aminocyclopropane-1-carboxylate deaminase [49, 72] to radioresistance [80] and Zn resistance [62]. The fact that members of *Methylobacterium* and *Flavobacterium* were found, might give an indication that these bacteria were selected for their use of toxic and derivatives present in the TGE and TGF.

As a corollary, we obtained evidence for the selection of microbial communities able to degrade phytotoxic compounds via lettuce seed germination test. This test assesses whether the level of phytotoxic compounds decreases as a result of microbial growth by measuring increase of seed germination. .The communities enriched in TGE as well as in TGF were able to remove the phytotoxicity. In further research (unpublished data) [115]it was proved that the bacterial strains *Stenotrophomonas maltophilia* (isolate 34), *Pseudomonas putida* (isolate 15), *Serratia plymuthica* (isolate 23), *Pseudomonas corrugata* (isolate 31), *Methylobacterium radiotolerans* (isolate 56), and *Agromyces aurantiacus* (isolate 95) could decrease the concentration of some of the toxic compounds present in TGF, such as 2-furaldehyde and pyrrole-2-carboxaldehyde, but not the phenol, 2-methoxyphenol and 2,6-dimethoxyphenol.

The above information is, to a certain degree, consistent with the expectation that degradable compounds that cause phytotoxicity might be transformed by the bacterial community selected. To deal with the phytotoxic compounds from TGE and TGF, bacteria should possess specific mechanisms to either break down or tolerate potentially toxic compounds. Moreover, we focused our attention on the bacteria but other microorganisms such as fungi could also be important for diminishment of toxicity.

This investigation on the colonization of TGE and TGF systems represents the first study that addresses the settlement of bacteria on torrefied grass fibers. After many enrichment steps, the final communities in both systems consisted of approximately 10

dominant bacterial species growing together on the substrates. Moreover, the communities of TGE and TGF were rather different. A decrease in phytotoxicity due to the microbial enrichment was observed in TGE as well as in TGF. However, many questions still remain. For instance, which bacteria present in the enriched community are involved in reducing phytotoxicity, and can they be applied to TGF to obtain a substrate suitable for plant growth?

Acknowledgements

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CHAPTER 3

Removal of phytotoxic compounds from torrefied grass fibres by plant-beneficial microorganisms

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Abstract

This research aimed to select microorganisms colonizing torrefied grass fibres (TGF) and simultaneously reducing the phytotoxicity which appeared after heat treatment of the fibres. Eighty-eight bacterial strains and one fungus, previously isolated from a sequential enrichment experiment on torrefied fibres and extracts were tested separately for their capacity to decrease phytotoxicity. Eleven of the bacterial strains and the fungus significantly reduced phytotoxicity. These organisms were checked for their ability to grow on agar containing phenol, 2-methoxyphenol, 2,6-dimethoxyphenol, 2-furalaldehyde, pyrrole-2-carboxaldehyde and furan-2-methanol as sole C-sources. The fungus F/TGF15 and the bacterial strain 66/TGF10 could grow on all six compounds. Strains 15/TGE5, 23/TGE5, 43/TGE20, 56/TGF10 and 95/TGF15 grew on two up to four compounds, and strain 72/TGF15 only on one compound. Strains 31/TGE5, 34/TGE5, 48/TGE20 and 70/TGF15 did not grow on any of the single toxic compounds. Gas chromatographic analyses of torrefied grass extracts determined which compounds were removed by the microorganisms. F/TGF15 was the only isolate depleting phenol, 2-methoxyphenol, 2-dihydrofuranone and pyrrole-2,5-dione-3-ethyl-4-methyl. Strains 15/TGE5, 23/TGE5, 31/TGE5 and 56/TGF10, and the fungus depleted 2-furalaldehyde, 2-furan-carboxaldehyde-5-methyl, pyrrole-2-carboxaldehyde, 5-acetoxymethyl-2-furaldehyde, and benzaldehyde-3-hydroxy-4-methoxy. These most promising candidates to colonize and simultaneously reduce the phytotoxicity of torrefied grass fibres were affiliated with *Pseudomonas putida*, *Serratia plymuthica*, *Pseudomonas corrugata*, *Methylobacterium radiotolerans*, and *Coniochaeta ligniaria*.

Introduction

Since the late 1970s, there has been a worldwide search for new peat substitutes, [95, 98]. Substitutes of peat are needed, on the one hand because of a general lack of peat moss resources in some countries and, on the other hand, due to the limitation of peat in the near future as a result of environmental constraints [1]. Different compounds have been investigated as potential substitutes of peat. Among them are cotton gin trash [87], vermicompost [131], wood fibres [38] and pine bark [128]. Recently, a new candidate for peat replacement within potting soil has also been suggested, namely torrefied grass fibres [115]. Torrefied grass fibres have good water holding capacity and they are a renewable substrate.

By exposing grass fibres to 240 °C for 1h, stabilization of the material and protection against decay are achieved. However, phytotoxic compounds such as phenol and phenolic compounds (2-methoxyphenol; 2, 6-dimethoxyphenol), 2-furalaldehyde (furfural),

pyrrole-2-carboxaldehyde and furan-2-methanol are produced in the process[115]. Furans and dehydration products thereof (2-furalaldehyde and zdr Nikihydroxymethyl furfural) are produced by dehydration of solubilised sugars. The release of phenolic material can arise from lignin depolymerization. Similar compounds have been found in steam-treated wheat straw exposed for different times at 210 °C, namely: extractable phenolics, tannin, 2-furalaldehyde and hydroxy-methyl-2-furalaldehyde [13]. Such chemical changes incurred during the torrefaction may have been responsible for the phytotoxic effect. Phenol and phenolic compounds have been found to be toxic for the germinability of maize (*Zea mays* L.) seeds [91] and tomato plants [6]. Even microorganisms are described to be inhibited by some of those toxic compounds [78, 130].

Following torrefaction, the grass fibres represent a microbiological vacuum, offering specific colonisable niches for microbes. This would allow a microbial community with beneficial properties, e.g. improving plant growth or suppressiveness against plant diseases, to be established. However, the phytotoxicity and, by inference, toxicity to microorganisms themselves, might pose limitations to this colonization process. We thus posed the question if microorganisms can transform the aforementioned toxic compounds and eventually grow on them, thus reducing their toxic effect. In previous work, we showed that specific bacterial communities could colonize torrefied grass fibres, resulting in a stable microbial population [115]. So far, only few bacteria, i.e. *Escherichia coli* (strains LYO1 and KO11), *Klebsiella oxytoca* (strain P2) can reductively detoxify furfural (5 and 10 mM) into furfuryl alcohol [23, 41, 73]. Bacterial strains from genera *Klebsiella, Enterobacter, Citrobacter, Edwardsiella* and *Proteus* and the ascomycete *Coniochaeta ligniaria* detoxify furfural (20 mM) and 5-hydroxymethyl furfural (5-HMF, 15 mM) into furfuryl alcolohol by co-metabolism [13, 70, 78]. Furfural and 5-HMF are released during acid pre-treatment of lignocelluloses biomass and they are toxic for microorganisms used for subsequent fermentation of lignocelluloses biomass. According to Boopathy *et al* (1993) the furfural and 5-HMF were not used as sole sources of carbon and energy, but they were transformed in the presence of glucose and peptone. The fungi *Pleurotus* spp. [118], *Pycnoporus cinnabarinus* and *Coriolopsis rigida* [6] were found to decrease or totally remove phenol from olive mill residues.

The aim of the current study was to investigate removal of phytotoxic compounds from torrefied grass fibres. For this purpose, reduction of phytotoxicity was checked among 88 bacterial strains and 1 fungus in a seed germination assay. A selection of 11 bacteria and 1 fungus was further tested for their ability to grow on six potential phytotoxic compounds, as well as to deplete phytotoxic compounds present in torrefied grass extract.

Materials and Methods

Microbial strains

Eighty-eight bacterial isolates and one fungus of different origin were assessed for their ability to remove phytotoxic compounds. Organisms were obtained from enrichment cultures in torrefied grass extract (TGE) and torrefied grass fibres (TGF) after an initial inoculation with a suspension from fireside soil, from TGE without a soil inoculum (AR), from TGE initially inoculated with an arable soil (WK3), and from TGE initially inoculated with forest soil (FR3) [115].All strains were maintained as pure cultures on R2A (Difco, Detroit, MI, USA) medium at 25 °C. Long-term storage was in glycerol stocks at -70 °C.

Torrefied grass fibre extraction

Extracts of grass fibre were prepared by the following protocol: 9.5 g of torrefied grass fibre was extracted at room temperature with 250 ml distilled water for 2 h using a magnetic stirrer at 700 rpm. Following the extraction, the suspension was paper-filtered to remove grass fibre particles, yielding TGE. Since the pH 6.5-7 is the optimum for most strains tested, the pH was adjusted to 6.8 with KOH. The TGE was then filter-sterilized (Millex GV bacterial filter Unit 0.22 µm Millipore) and stored at 4 °C.

Phytotoxicity diminishment

The 88 bacterial isolates and the fungus were grown in 20 ml liquid medium containing (1:1) torrefied grass extract (TGE) and minimal salt medium (MSM). MSM ingredients were K_2HPO_4 1.4 g/l, KH_2PO_4 1.4g/l, $MgSO_4.7H_2O$ 0.1 g/l, $(NH_4)_2SO_4$ 1 g/l, $MnSO_4.7H_2O$ 0.001 g/l, $FeSO_4.7H_2O$ 0.01 g/l. The isolates were grown as single strains during 5 days at 25 °C and 180 rpm using a horizontal shaker. Thereafter, the cells were removed from the TGE: MSM culture by centrifugation (9.400 x *g*, 10 min) and filter-sterilization (Millex GV bacterial filter Unit 0.22 µm Millipore). The filtrate was used in a germination assay with lettuce seeds (*Lactica sativa*, cultivar "Erika", Enza Zaden, Enkhuizen, the Netherlands) to monitor the reduction of phytotoxic compounds; 250 µl was used per well of 24-well plates (Greiner Bio-one, CELLSTAR® Germany). The test was performed in six wells in a randomized block design, each with 5 seeds. After 48 h, the number of seeds that had germinated was counted. As controls, distilled H_2O, TGE: MSM (1:1), and TGE were used.

Growth of isolates on phytotoxic compounds

Eleven bacterial strains and one fungal strain with best results in the seed germination assay were evaluated for their potential growth on phytotoxic compounds in TGF. Six model compounds, i.e. phenol (QBiogene, USA), pyrrole-2-carboxaldehyde, 2-methoxyphenol, 2,6-dimethoxyphenol, 2-furaldehyde and furan-2-methanol (Alfa Aesar

GmbH & Co.KG, Germany) were used as sole carbon sources in MSM agar medium. Agar plates with 0.1% and 0.01% of each compound were prepared in duplicates. Bacterial strains were inoculated in strikes on the plates and their growth on the agar was evaluated. MSM agar medium without any carbon was used as negative control and MSM agar medium containing 1% glucose was the positive control.

Removal of phytotoxic compounds

Eleven bacterial strains and one fungal strain, with best results in the seed germination assay, were cultured on TGE: MSM liquid medium at 25 °C , 160 rpm. Bacterial strains were cultured for 5 days and the fungus for 7 days. To determine growth, dilution plating was done on R2A media (Difco, Detroit, MI, USA). Thereafter, the bacterial and fungal cells were removed from TGE: MSM suspension by centrifugation (9.400 x *g*, 10 min) and the supernatants were filter-sterilized (Millex GV bacterial filter Unit 0.22 µm, Millipore). The resulting filtrate was assessed in the lettuce seed germination assay as described previously and analyzed with gas chromatography (GC MS).

GC MS analyses

Two ml from the TGE filtrate and 2 ml of dichloromethane were mixed together and vortexed for about 10 sec, until a homogeneous solution was obtained. After centrifugation (1.200 x *g*, 5 min), the upper inorganic layer was discarded and the organic layer transferred to a clean vial and passed over a short column (Pasteur capillary pipette filled with siliconized glass wool and anhydrous Na_2SO_4). After this, the clean filtrate was collected and stored at -20 °C until measurements.

Two µl of the filtered organic phase were injected in splitless mode into the injection port of a gas chromatograph (5888 series II, Hewlett-Packard GMI, USA) coupled to a mass spectrometer (model 5972A, Hewlett-Packard GMI, USA) with a Zebron ZB-5ms column (30 m × 0.25 mm I.D. × 0.25 µm film thickness) (Phenomenex, USA). The carrier gas was He (1 ml/min). The oven was programmed at an initial temperature of 45 °C for 1 min, with a ramp of 10 °C per min to 310 °C, and final time of 8.5 min. The injection temperature was 250 °C, and the detection temperature was 290 °C. Peaks were tentatively identified by comparison of the spectra to commercial databases as well as to reference compounds: phenol (QBiogene, USA), pyrrole-2-carboxaldehyde, 2-methoxyphenol, 2,6-dimethoxyphenol, 2-furaldehyde and furan-2-methanol (Alfa Aesar GmbH & Co.KG, Germany).

Data from bacteria or fungus incubated in TGE: MSM were expressed as percentage of the concentration of compounds present in the TGE: MSM control without microbial growth.

Statistics

To evaluate the data from the seed germination assay and the compounds from GC MS, analysis of variance (ANOVA) was applied with Genstat release 9.2 (Rothamsted Experimental station, Harpenden, UK). Least significant differences were calculated for P=0.05.

The concentration of the compounds which had been measured with GC MS in the TGE samples incubated with the different bacteria and the fungus, were correlated with the germination of lettuce seeds and with growth of the bacteria in TGE: MSM by multivariate analyses with the statistical program CANOCO release 4.5 (Ter Braak, 1995). The concentration of the compounds was log transformed and analyzed with redundancy analysis (RDA) since the structure of the data was linear. Scaling of the figures was focused on inter-species correlations. Significance of the environmental factors (percentage germination and CFU) was analyzed with Monte Carlo permutation based on 499 random permutations.

Results

Phytotoxicity diminishment

The 88 bacterial and 1 fungal strains of different origin (TGE5, TGE20, TGF10, TGF15, AR, WK3, and FR3) were tested for their capability to break down, in an overall fashion, the phytotoxic compounds present in TGE. Five out of 15 isolates from TGE5, namely 15/TGE5, 23/TGE5, 26/TGE5, 31/TGE5 and 34/TGE5, showed significant enhancement of seed germination (Table 1), improving germination rates with about 25% (Fig 1).

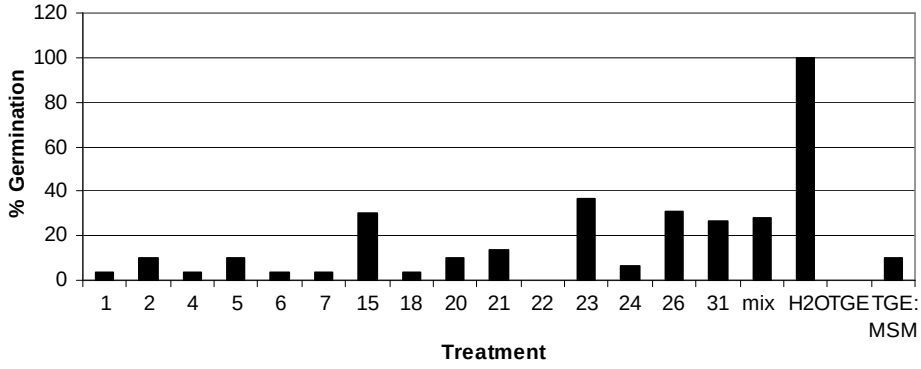

Fig. 1 Lettuce seed germination assay with 15 isolates from TGE5 after incubation in TGE:MSM. “Mix” is the combination of isolates 15/TGE5, 23/TGE5, 26/TGE5 and 31/TGE5. Three controls were applied: H_2O, torrefied grass extract (TGE), and TGE:MSM. Least significant difference is 18 (P=0.05).

Table 1 Isolates decreasing phytotoxicity in torrefied grass extract (TGE) assessed in a lettuce seed germination assay

Strain	Closest affiliate	Gen bank accession numbers	Nr of tests with germination improvement *	Total number of tests
15/TGE5	*Pseudomonas putida*	EU293384	4	4
23/TGE5	*Serratia plymutica*	EU293380	3	4
26/TGE5	*Flavobacterium denitrificans*	EU450835	1	2
31/TGE5	*Pseudomonas corrugata*	EU293383	3	5
34/TGE5	*Stenotrophomonas maltophilia*	EU293366	2	2
43/TGE20	*Rhizobium radiobacter*	EU293379	2	2
48/TGE20	*Flavobacterium denitrificans*	EU293378	2	2
56/TGF10	*Methylobacterium radiotolerans*	EU293389	3	3
66/TGF15	*Leifsonia xyli subsp. xyli*	EU293373	2	2
70/TGF15	*Mycobacterium anthracenicum*	EU293382	2	2
72/TGF15	*Agrococcus casei*	EU293376	3	3
95/TGF15	*Agromyces aurantiacus*	EU293381	4	4
F/TGF15	*Coniochaeta ligniaria*	EU450836	2	2

***** Number of tests with a significant improvement of lettuce seed germination (P=0.05)

Since the positive result of 26/TGE5 was only obtained once, this strain was not used for further tests. Two out of 15 strains obtained from TGE20, namely isolates 43/TGE20 and 48/TGE20, one out of 6 isolates from TGF10, namely isolate 56/TGF10, and five out of 10 from TGF15, namely isolates 66/TGF10, 70/TGF15, 72/TGF15, 95/TGF15 and the fungus F/TGF15 significantly enhanced seed germination as well (Table 1). Two out of 22 isolates from WK3 (12/WK3 and 19/WK3) and one out of 12 from FR3 (4/FR3) diminished phytotoxicity, giving significant increases of germination rates (data not shown). No significant improvements were found for the 8 AR isolates. In general, seed germination was improved about 25-35% by single strains. When the four most efficient single isolates from TGE5 were mixed and tested, this yielded a similar enhancement of seed germination as any of the single strains, i.e. 25-35% germination (Fig.1). The water control consistently yielded 100% germination, while TGE yielded 0% germination, clearly

showing the consistency of toxicity. The control TGE: MSM yielded a lightly higher germination percentage than the TGE control due to the dilution of TGE.

Growth of isolates on phytotoxic compounds

Strains 15/TGE5, 23/TGE5, 31/TGE5, 34/TGE5, 43/TGE20, 48/TGE20, 56/TGF10, 66/TGF10, 70/TGF15, 72/TGF15, 95/TGF15, and F/TGF15 were used for growth tests on potentially phytotoxic compounds. The fungus F/TGF15 and the bacterial strain 66/TGF10 could grow on all six compounds if 0.01% was added to the agar. Strains 15/TGE5, 23/TGE5, 43/TGE20, 56/TGF10 and 95/TGF15 grew on 2 up to 4 of the compounds (Table 2).

Strain 72/TGF15 grew on only one compound. However, strains 31/TGE5, 34/TGE5, 48/TGE20 and 70/TGF15 did not grow on any of the single toxic compounds. All strains grew on the positive control with glucose as carbon source and did not grow on the negative control without any carbon source (data not shown).

None of the isolates grew on 0.1% of the compounds, with 1 exception, F/TGF15 which grew on 0.1% 2,6–dimethoxyphenol. Concentrations of the model compounds were between 6.8 and 28 mg/kg TGF, which corresponds 0.0007 and 0.003%. Thus, the 0.1% concentrations were much higher than the concentrations present in TGF.

Removal of phytotoxic compounds

Figure 2 provides an example of the compounds present in TGE, as well as in TGE inoculated with strain 15/TGE5. Several peaks have disappeared after growth of this isolate.

Most of the bacteria and the fungus multiplied in TGE:MSM and CFU were detected from log 5 up to log 9.4. The data of the few isolates that, for unknown reason, did not grow during the incubation in TGE:MSM were excluded from multivariate statistical analysis presented in Fig. 3. This figure illustrates the correlation between the germination rate of lettuce seeds, CFU microbial numbers and 14 most relevant compounds that were detected in TGE. Most duplicate samples had very similar values and both axes as well as the two explanatory factors (CFU, % germination) were significant ($P = 0.002$). Significant reductions of several of the potentially phytotoxic compounds in TGE were measured. The ordination plot Fig. 3 shows that the fungal isolate F/TGF15 and the bacterial isolate 56/TGF10 had the highest increase in seed germination (isolates are in the same direction as the vector % germination). Most compounds correlated negatively with the % germination, indicating that the decrease of these compounds enhances germination of the lettuce seeds. Vectors of some of the compounds had an almost identical direction, i.e. compounds with the retention time 4.57, 6.46, 11.62 and 12.88 min, showing that their depletion in the different samples was similar. The most active strains in removing toxic

compounds from TGE were F/TGF15, and 56/TGF10. Strains 23/TGE5, 34/TGE5, 15/TGE5 and 31/TGE5 were removing or depleting several compounds as well. Strains 43/TGE20, 72/TGF15 and 95/TGF15 were the least effective in removing compounds.

Remarkably, the compound 2-furanmethanol with retention time 4.80 min was absent in the TGE:MSM control (Fig. 2) and increased in some samples with bacterial growth (Fig. 3), indicating that this compound arises due to bacterial growth. It is expected that this compound is a break down product of one of the potentially phytotoxic compounds that were degraded.

Figure 4 shows the removal of several of the compounds by the separate strains. Compounds 2-furalaldehyde and pyrrole-2-carboxaldehyde were depleted by strains 15/TGE5, 23/TGE5, 31/TGE5, 56/TGF10 and F/TGF15 and partially by 34/TGE5 (Fig. 4, upper graph). Similarly, 2-furan-carboxaldehyde-5-methyl, 5-acetoxymethyl-2-furaldehyde, and benzaldehyde-3 hydroxy-4-methoxy were depleted by the same strains (data not shown). The fungus F/TGF15 was the only strain that removed phenol, 2-methoxyphenol, (Fig. 4, middle) 2-dihydro-furanone and pyrrole-2,5-dione-3-ethyl-4-methyl (data not shown). Ethanone-1-(4-hydroxy-3-methoxy phenyl) was only removed by the strains 56/TGF10 and F/TGF15 and partially, but significant, decreased by the strains 15/TGE5 and 23/TGE5 (Fig. 4, lower graph). Benzopyran-2-one was not removed by any of the tested strains, however, isolate 15/TGE5, 23/TGE5, 56/TGF10, 95/TGF15 and F/TGF15 reduced the concentration significantly (Fig. 4, lower graph).
Since two major groups of compounds were detected to be present in TGE, namely 2-furaldehyde and its derivatives and phenol and its derivatives, 2 representative compounds from those two groups were accurately quantified. The actual concentrations present in the TGE:MSM were 0.68 mg/l for 2-furaldehyde and 0.07 mg/l for 2-methoxyphenol.

Discussion

In this study, we investigated a selection of strains for their capability to remove or transform phytotoxic compounds present in torrefied grass, a renewable substrate that can be used as an ingredient of potting soil. For that purpose, 88 strains from different origins, that had previously been isolated from enriched torrefied grass extracts and fibres [115], were tested. A lettuce seed germination assay monitored the reduction of toxicity to lettuce seeds in TGE in an overall way. Several of the isolates, including the fungus *Coniochaeta ligniaria*, significantly improved lettuce seed germination. The phytotoxicity-diminishing microorganisms belonged to different taxonomic groups, indicating a broad taxonomic spread of the capacity to reduce phytotoxicity across bacteria; i.e. *Pseudomonadaceae, Burkholderiaceae, Enterobacteriaceae, Methylobacteriaceae, Flavobacteriaceae, Microbacteriaceae, Rhizobiaceae, Mycobacteriaceae*, and *Xanthomonadaceae*.

Table 2

Growth of selected isolates on agar with potential phytotoxic compounds used as a sole C source (concentration 0.01%)

Strain	Closest affiliate	Phenol	2-Methoxy-phenol	2-Furaldehyde	2,6-Dimethoxyphenol	Pyrrole-2-car-boxaldehyde	Furan-2-methanol
15/TGE5	*Pseudomonas putida*	1	0	1	0	0	1
23/TGE5	*Serratia plymutica*	1	0	1	0	1	1
31/TGE5	*Pseudomonas corrugata*	0	0	0	0	0	0
34/TGE5	*Stenotrophomonas maltophilia*	0	0	0	0	0	0
43/TGE20	*Rhizobium radiobacter*	1	0	1	0	0	0
48/TGE20	*Flavobacterium denitrificans*	0	0	0	0	0	0
56/TGF10	*Methylobacterium radiotolerans*	0	0	2	0	0	1
66/TGF10	*Leifsonia xyli subsp. xyli*	2	2	1	1	1	1
70/TGF15	*Mycobacterium anthracenicum*	0	0	0	0	0	0
72/TGF15	*Agrococcus casei*	0	0	0	0	0	1
95/TGF15	*Agromyces aurantiacus*	1	0	1	0	0	1
F/TGF15	*Coniochaeta ligniaria*	2	2	1	2	1	2

0 = no visual growth, 1 = tiny visual colonies of 0.1 to 0.3 mm, 2 = colonies of > 0.3 mm

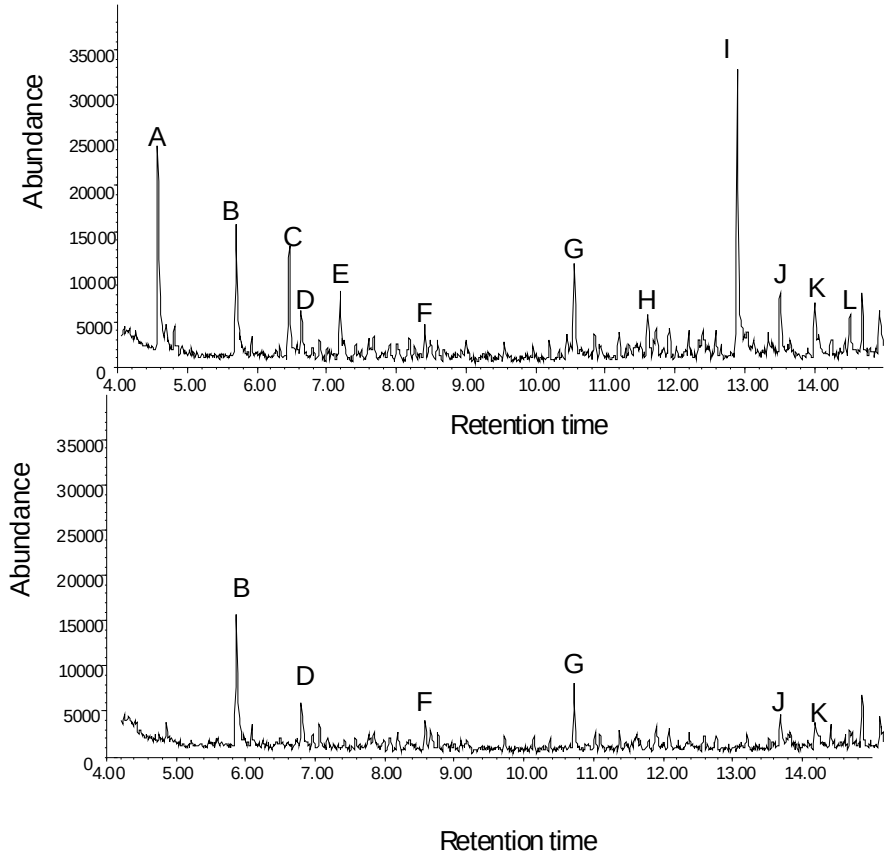

Fig. 2 Chromatographic peaks present in TGE:MSM without microorganisms (control) (upper panel) and TGE:MSM incubated with isolate 15/TGE5 during 5 days (lower panel). Compounds with retention time in minutes between brackets: (A) 2-furalaldehyde (4.57), (B) 2-dihydrofuranone (5.70), (C) 2-furan-carboxaldehyde-5-methyl (6.46), (D) phenol (6.63), (E) pyrrole-2-carboxaldehyde (7.19), (F) 2-methoxyphenol (8.41), (G) pyrrole-2,5-dione-3-ethyl-4-methyl (10.56), (H) 5-acetoxymethyl-2-furaldehyde (11.62), (I) benzaldehyde-3 hydroxy-4-methoxy (12.90), (J) benzopyran-2-one (13.51), (K) ethanone-1-(4-hydroxy-3-methoxyphenyl)(14.01),(L)5-acetylaminomethyl-4-amino-2-methylpyrimidine (14.52).

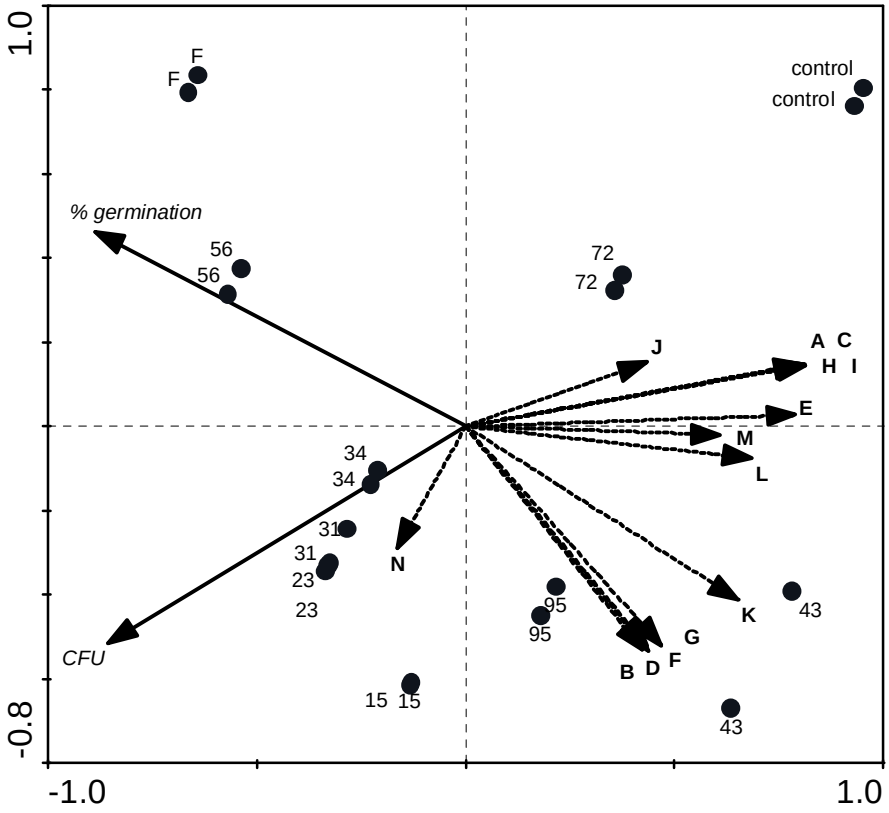

Fig. 3 Ordination plot of duplicate TGE:MSM samples incubated with the different strains (points with strain numbers 15/TGE5, 23/TGE5, 31/TGE5, 34/TGE5, 43/TGE20, 56/TGF10, 72/TGF15, 95/TGF15, F/TGF15) and the control, the presence of distinct compounds (dotted vectors) and germination of lettuce seeds and growth of the bacteria in TGE: MSM as explanatory factors (filled vector). The plots were generated by RDA. Values on the axes indicate the percentage of total variation of the compounds explained by each axis. Vectors pointing in the same direction are positively correlated and those pointing in opposite directions are negatively correlated. Compounds with retention time in minutes between brackets: (A) 2-furalaldehyde (4.57), (B) 2-dihydrofuranone (5.70), (C) 2-furan-carboxaldehyde-5-methyl (6.46), (D) phenol (6.63), (E) pyrrole-2-carboxaldehyde (7.19), (F) 2-methoxyphenol (8.41), (G) pyrrole-2,5-dione-3-ethyl-4-methyl (10.56), (H) 5-acetoxymethyl-2-furaldehyde (11.62), (I) benzaldehyde-3 hydroxy-4-methoxy (12.90), (J) benzopyran-2-one (13.51), (K) ethanone-1-(4-hydroxy-3-methoxyphenyl) (14.01), (L) 5-acetylaminomethyl-4-amino-2-methylpyrimidine (14.52), and (N) 2-furanmethanol (4.80).

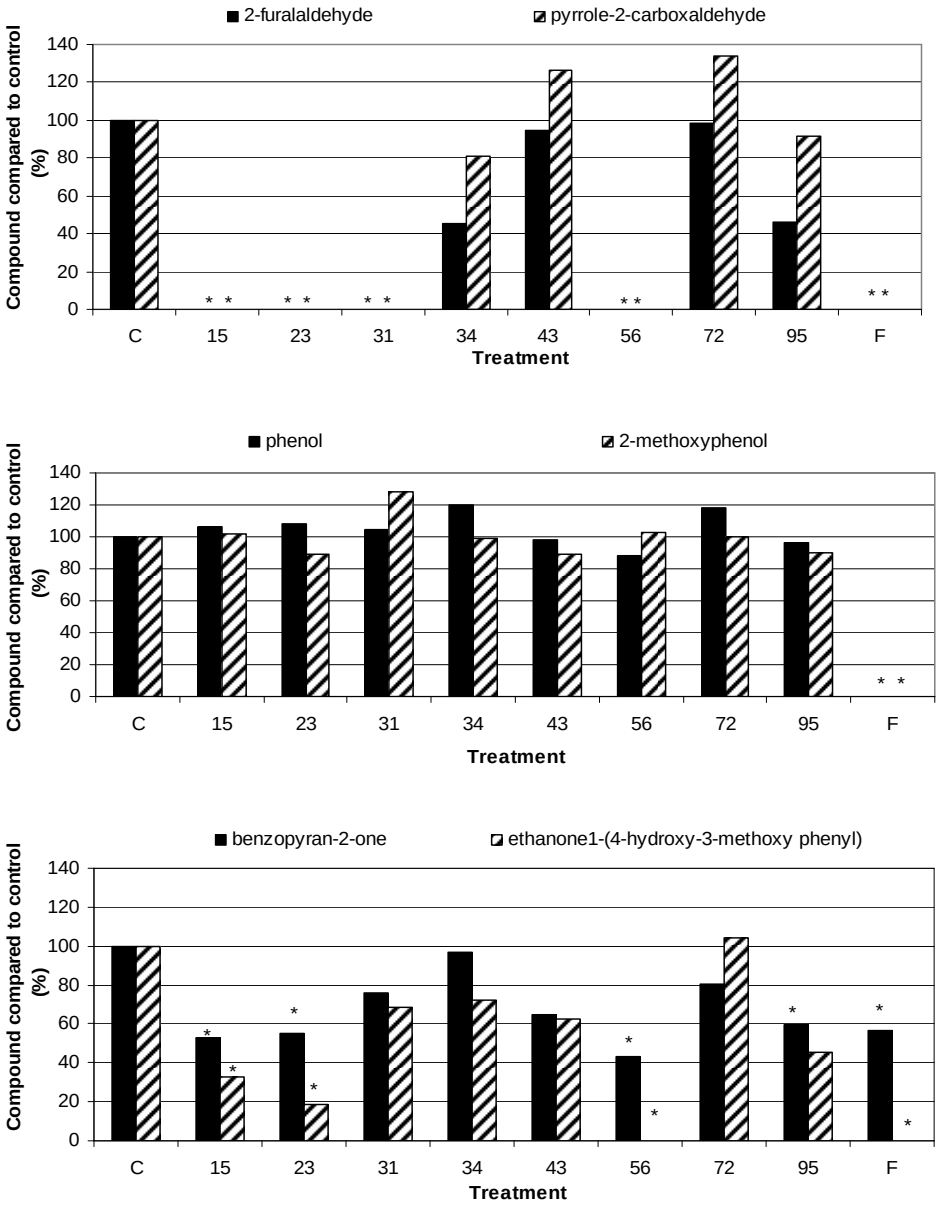

Fig. 4 Removal of compounds from TGE by the bacterial and fungal strains 15/TGE5, 23/TGE5, 31/TGE5, 34/TGE5, 43/TGE20, 56/TGF10, 72/TGF15, 95/TGF15, F/TGF15 as compared to the control (C), which was TGE:MSM without a microbial inoculant. Bars with an asterisk are significantly (P=0.05) lower than the control value.

These phytotoxicity-diminishing bacteria and the fungus *Coniochaeta ligniaria* are highly interesting candidates to be used for the re-colonization of torrefied grass fibres, to decrease phytotoxicity as well as to create a complex and stable microbial community in the substrate.

In a previous study, the following potentially phytotoxic compounds were measured in the torrefied grass fibres: phenol (11mg/kg), phenolic compounds such as 2-methoxyphenol (10.8 mg/kg), 2,6-dimethoxyphenol (10.2 mg/kg), 2-furalaldehyde (27.9 mg/kg), pyrrole-2-carboxaldehyde (6.8 mg/kg) and furan-2-methanol (8.6 mg/kg)[115]. Since the above-mentioned seed germination assay is a 'black box' strategy, it was not known which of the compounds were depleted by the microorganism. To obtain knowledge on separate phytotoxic compounds, additional experiments were performed: (1) growth of the selected isolates on toxic compounds used as single carbon sources and (2) assessment of removal of compounds present in TGE by the growth of selected isolates. For the latter, compounds were measured by gas chromatography mass spectrometry (GC MS).

The fungus, which was one of the most effective strains in improving seed germination, grew on each of the six toxic compounds tested and removed most of the potentially phytotoxic compounds present in TGE, including phenol and 2-methoxyphenol, as shown by GC MS analyses. Phenol and phenolic compounds are toxic for maize seeds [91] and tomato plants [6]. Thus, depletion of these compounds from TGE can be expected to increase seed germination of lettuce, which is relatively sensitive for toxic compounds. *C. ligniaria* is a soil-born fungus, commonly occurring on decaying wood and wood pulp [22]. Nilsson [78] isolated a strain of *C. ligniaria,* NRRL30616 from soil that metabolizes many inhibitory compounds such as phenol, 2-furaldehyde, 5-HMF and reduces the concentration of 2-furalaldehyde, HMF (furan dehydration products) and acetate in corn stover hydrolysates essentially to zero.

Six of the bacteria could break down or decrease the concentration of several of the compounds present in TGE. These isolates 15/TGE5, 23/TGE5, 31/TGE5, 34/TGE5 56/TGF10 and 95/TGF15 were respectively affiliated with *Pseudomonas putida, Serratia plymuthica, Pseudomonas corrugata, Stenotrophomonas maltophilia, Methylobacterium radiotolerans* and *Agromyces aurantiacus*. Intriguingly, the first four isolates were all gamma-proteobacteria originating from the same treatment; they were isolated from the fifth enrichment step in TGE. Isolates 15/TGE5, 23/TGE5 and 56/TGF10 and 95/TGF15 were able to grow on several of the potentially phytotoxic compounds as sole carbon source. However, isolates 31/TGE5 and 34/TGE5 did not grow on any of those compounds as sole carbon source. It is possible that the model compounds, admittedly provided in relatively high concentrations compared to the natural substrate, were simply toxic to the strains, thus limited the ability for growth. Co-metabolism is another likely explanation for the discrepancy between the lack of growth on sole carbon sources and the ability of

depleting such carbon sources in a mixed substrate such as TGE. In a previous study, it was observed that the strains often did not grow on formic acid and acetic acid as a sole carbon source, but did grow on the combination [115]. For *S. maltophilia* it has been described that the utilization of one substrate has a considerable effect on the utilization of another one, when studying consumption of aniline and glucose [134].

The most promising bacterium for colonization of torrefied grass fiber is the isolate 56/TGF10, affiliated with *M. radiotolerans*. It was most active in increasing seed germination, improving the germination with about 35%. This isolate not only removed 2-furaldehyde, 2-furancarboxaldehyde-5-methyl, pyrrole-2-carboxaldehyde, 5-acetoxymethyl-2-furaldehyde, but it was also able to deplete fully the compounds ethanone1-(4-hydroxy-3-methoxy phenyl) and 5-acetylaminomethyl-4-amino-2-methyl pyrimidine, where the other 4 bacterial strains only reduced their concentration. Interestingly, this isolate was from the enrichment of fibres instead of extract.

It is still not fully clear which of the compounds present in TGF and TGE are responsible for the phytotoxicity. Phenolic compounds certainly play a role, and they are depleted by the fungus, but not by the bacteria. Therefore, the effect of the bacteria should be due to depletion of one or more of the other compounds present in TGE. The phytotoxicity of 2-furalaldehyde derivatives is unclear. 2-Furalaldehyde has been reported to be toxic to rumen microbes [63], although Castro *et al* [13] disproved this. Phytotoxicity of the TGE was found to be concentration dependent, since phytotoxicity decreased after dilution of the extract. It can also be the combination of all the compounds and its derivatives causing the phytotoxicity. Thus, testing the separate compounds for their phytotoxic effect on lettuce seeds is of limited value. Since the lettuce seed germination was improved at most about 35%, TGF will most likely be applied in future experiments in a combined mix with other potting soil ingredients.

In summary, the fungus *C. ligniaria* removed almost all potentially phytotoxic compounds present in TGE, including phenolic compounds. Additionally, four interesting bacterial isolates were selected to be capable of removing or decreasing concentrations of several phytotoxic compounds in TGE, however, not the phenolic ones. These four bacteria, i.e. *P. putida, S. plymuthica, P. corrugata,* and *M. radiotolerans* are all known for their antagonistic properties against plant pathogens [40, 57, 133]. Thus, the results of our research showed that a microbial community with the capacity to remove phytotoxic compounds from TGE and the potential to promote plant-growth or plant-health was selected. This is an important step in the development of a renewable substrate containing a plant beneficial microflora.

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CHAPTER 4

Colonization of torrefied grass fibers by plant-beneficial microorganisms

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Abstract

This study aimed to assess the colonization of thermally treated (i.e. torrefied) grass fibers (TGF), a new prospective ingredient of potting soil. Eleven bacterial strains and one fungus, *Coniochaeta ligniaria* F/TGF15, all isolated from TGF or its extract after inoculation with a soil microbial community, were tested for their ability to colonize TGF. Surprisingly, none of these bacteria were able to directly colonize TGF either as single inoculants or as a consortium. Furthermore, bacterial persistence or growth in TGF was not improved by the addition of nutrients or a surfactant. Only extensive washing of the substrate, presumably removing bacteriostatic or bactericidal compounds, allowed bacterial growth occur on the fibers. Strikingly, the fungal strain consistently colonized TGF up to high densities (up to 10^{10} CFU per g dry TGF). Given the unique capacity of this fungus to degrade toxic compounds including phenols, TGF was colonized with it for different periods of time, after which a consortium of seven selected bacterial isolates was added. Co-presence of the fungus, or 3 and 24 hours pre-colonization with it, were insufficient to create a habitable environment for the bacterial consortium. However, fungal pre-colonization of minimally 3 days allowed the bacterial consortium to colonize the TGF at numbers up to 10^{9}-10^{10} CFU per g dry substrate. The resultant bacterial community consisted of at least four strains, i.e. *Pseudomonas putida* 15/TGE5, *Serratia plymuthica* 23/TGE5, *Pseudomonas corrugata* 31/TGE5 and *Methylobacterium radiotolerans* 56/TGF10, as shown by PCR of colonies on plates and PCR-DGGE profiling. Two persisters, *S. plymuthica* 23/TGE5 and *P. corrugata* 31/TGE5, were highly antagonistic towards several phytopathogenic fungi. Thus, a microbial community with plant-beneficial potential can be established on TGF, provided that the fungus *C. ligniaria* F/TGF15 first creates habitable space in the matrix.

Introduction

Torrefied grass fibers (TGF) have been suggested as a new substrate for plant growth, which might gradually replace peat in potting soil. Gras [115]s is grown worldwide and it represents a renewable substrate, in contrast to peat. By exposing dried grass fibers to a heat treatment, i.e. torrefaction, the fibers are transformed into a stable matrix which is fairly protected against decay. Torrefied grass fibers have a high water holding capacity, which is comparable to that of peat. However, a disadvantage is the occurrence of phytotoxic compounds after the heat treatment, which on the other hand may be decreased by microbial activity [115, 116].

The conditions of sterility of the TGF just after the heat treatment offer an ideal situation for the introduction of selected microorganisms with desired properties. Thus, survival of introduced microorganisms will not be hampered by competition with an autochthonous microbial population. A crucial question which arose is: how should an ideal microbial community be composed, i.e. what are the requirements concerning the community composition leading to a stable community, and, what are the desired properties of the individual microorganisms that make up the consortium? A first requirement might be the microbial ability to break down the phytotoxic compounds that may be present on the TGF. Finally, microorganisms that are equipped with plant-beneficial properties would give the substrate an added value. Plant-growth-promoting bacteria, as well as plant pathogen inhibiting microorganisms are prime candidates to make part of the microbial consortium.

A stable microbial community in TGF should, theoretically at least, consist of a consortium comprising both *r* and *K* strategists. Novel niches provided by the TGF system will first be colonized by a limited number of “cochroach-like” species with high growth rates and high fecundity (*r* strategists). These organisms are highly opportunistic, showing little specialization. They are expected to rapidly utilize the easily available substrates [28]. Early stages of colonization of novel habitats are thus often characterized by low diversity and evenness values [125]. With time, an increasing number of pre-existing or newly-formed niches will be filled, and increasing demands will be placed on the competitive abilities of individual populations since easily-available substrates become depleted. As a result, the diversity and evenness levels in the system will increase. In the later stages of colonization, *K* strategists, defined by their lower growth rates and fecundity, may establish in the community [28]. Thus, it is hypothesized that stable and mature microbial communities should comprise microorganisms of different ecophysiological types, including *r*- as well as *K*- strategists.

The most abundant easily-available carbon sources present in the TGF are acetic acid and formic acid [116]. These compounds can theoretically serve as nutrient and energy sources for many microorganisms. Moreover, phytotoxic compounds, formed as a side effect of the heating process, are present. Hence, micro-organisms that utilize these compounds are ideal candidates to be the first colonizers of the substrate.

In this study, we aimed to colonize TGF with a microbial consortium assembled on the basis of assumptions about ecological behavior and function. Thus, we aimed for a consortium that is able to:

(1) successfully colonize TGF and persist on it,

(2) reduce the phytotoxicity of the substrate, and

(3) have plant-beneficial properties.

Thus, 11 bacterial and one fungal strain were selected among - in total - 88 strains which originated from enrichment cultures in TGF and its extract [115, 116]. The criteria used to select these strains were: (1) the capacity to improve lettuce seed germination in TGF extract, (2) the capability to grow on and transform several phytotoxic compounds present in TGF, (3) growth on acetic acid and/or formic acid as the sole carbon source(s). In addition, we took into consideration these being either *r* or *K* strategists as well as their capacity to inhibit phytopathogenic fungi.

Material and methods

Bacterial isolates

Eleven bacterial strains were obtained from sequential enrichment cultures [115]. They belonged to the α-proteobacteria (*Rhizobium radiobacter* 43/TGE20, *Methylobacterium radiotolerans* 56/TGF10), γ-proteobacteria (*Pseudomonas putida* 15/TGE5, *Serratia plymuthica* 23/TGE5, *Pseudomonas corrugata* 31/TGE5, *Stenotrophomonas maltophilia* 34/TGE5) and the actinobacteria (*Leifsonia xyli subsp. xyli* 66/TGF10, *Mycobacterium anthracenicum* 70/TGF15, *Agrococcus casei* 72/TGF15, and *Agromyces aurantiacus* 95/TGF15), and the Flavobacterium–Cytophaga group (*Flavobacterium denitrificans* 48/TGE20). They were all capable to enhance lettuce seed germination [115], but they differed in characteristics such as: 1) the capability to utilize or deplete phytotoxic compounds from the extract of TGF, 2) the utilization of acetic acid and/or formic acid as carbon sources, 3) their presumed ecological strategy being *r* or *K* strategist, and 4) their origin (see Table 1).

Each strain was grown on R2A (Difco, Detroit, MI, USA). Short-term storage was at 4 ºC and long-term storage in 20% glycerol at -80 ºC.

The fungus Coniochaeta ligniaria F/TGF15

C. ligniaria F/TGF15 was isolated by sequential enrichment on TGF and identified by its 18S ribosomal RNA gene sequence [115]. *C. ligniaria* is an ascomycete (former genus name is *Phialophora*), which degrades lignocelluloses and is often found on woody substrates. It produces cream-colored to orange colonies with purplish brown tinges in the centre [22, 53]. *C. ligniaria* is a slow-growing organism. The fungal mycelium is formed after 10 days or more. This isolate gave significant enhancement of lettuce seed germination and can utilize many of the phytotoxic compounds present in TGF [115] (see Table 1). The fungus was maintained on PDA medium (Potato Dextrose Agar 39 g/l, Oxoid, London, UK) at 20 ºC in the dark.

In vitro antagonism of selected TGF bacteria towards a range of fungi

Antagonistic activity of selected bacterial strains towards several fungi was determined in an *in vitro* assay on PDA. Three fungi isolated from TGF, namely *Fusarium sp., Penicillium sp., Rhizopus sp.*, six phytopathogenic fungi (namely two strains of *Fusarium oxysporum* sp. *radicis lycopersici*, *Pythium ultimum, Pythium aphanidermatum*, *Rhizoctonia solani*, *Verticillium dahliae)* and one antagonistic fungus, namely *Trichoderma harzianum*, were assessed in this test. They were grown on PDA medium for a period of 2 to10 days at 20 ºC. A suspension of hyphal fragments and/or spores from each fungal strain was obtained by adding 10 ml sterile distilled water to the Petri dish. After scraping with a spatula, the suspension was diluted 10-fold in 0.85% NaCl. Aliquots (500 µl) of diluted suspension were added to Petri dishes and mixed with 10 ml PDA just before solidifying. The bacterial strains were inoculated at the edges of the inoculated Petri dishes. Each combination was performed in two replicates. After 5 days of incubation at 20 ºC, inhibition zones around the inoculated bacteria were measured.

Preparation of TGF for colonization experiments

Grass was collected in August 2003 from unfertilized grassland on a peaty clay soil near Wageningen, The Netherlands, as described before [115]. Samples were torrefied in a preheated Carbolite muffle furnace (Model CWF 1100) at 240 ^{o}C during 1 h. The pH of the thus prepared TGF was 5.4.

For all colonization experiments described below, TGF samples of 0.5 g were placed in 50-ml vessels and sterilized by gamma-irradiation (25 kGray). Subsequently, 1.6 ml of inoculant suspension prepared as described below was added, thus establishing 60 % of the substrate's water holding capacity. The system thus established was homogenized with a sterile needle and incubated at 25 ºC in the dark. Treatments in all experiments were done in two replicates.

Microbial inocula

Freshly-grown bacterial cells were harvested from R2A medium by adding 5 ml mineral salt medium (MSM: K_2HPO_4 1.4 g/l, KH_2PO_4 1.4g/l, $MgSO_4.7H_2O$ 0.1 g/l, $(NH_4)_2SO_4$ 1.0 g/l, $MnSO_4.7H_2O$ 0.001 g/l, and $FeSO_4.7H_2O$ 0.01 g/l) to the Petri dishes. The optical density (OD_{600}) of the suspension was adjusted to 0.1 with MSM using a Beckman DU® 530 Life Science UV/Vis spectrophotometer. A 1000-fold dilution was then made to obtain a suspension which contained approximately 10^5 CFU per ml .

The fungal strain *C. ligniaria* F/TGF15 was grown on PDA plates for 12 days at 20 ^{o}C. Then, 10 ml MSM were added per plate and fungal tissue and spores were scraped off with a spatula. The OD_{600} of this suspension was adjusted to 0.1 with MSM and the

Table1. Characteristics of isolates

Isolate Nr.	Name of closest affiliate	Genbank accession Nr.	Origin	Nr. of successful germination assays/ total [a]	R or K strategist	Taxonomic group	Nr. of toxic compounds used [b]	Growth on A, F, A+F [c]
15/TGE5	*Pseudomonas putida*	EU293384	TGE5	4 / 4	r	Gram -	3	A+F
23/TGE5	*Serratia plymutica*	EU293380	TGE5	3 / 4	r	Gram -	4	A, A+F
31/TGE5	*Pseudomonas corrugata*	EU293383	TGE5	3 / 5	r	Gram -	2	F, A+F
34/TGE5	*Stenotrophomonas maltophilia*	EU293366	TGE5	2 / 2	r	Gram -	0	A+F
43/TGE20	*Rhizobium radiobacter*	EU293379	TGE20	2 / 2	r	Gram -	2	A, F, A+F
48/TGE20	*Flavobacterium denitrificans*	EU293378	TGE20	2 / 2	r	Gram -	0	A+F
56/TGF10	*Methylobacterium radiotolerans*	EU293389	TGF10	3 / 3	K	Gram -	2	F, A+F
66/TGF10	*Leifsonia xyli subsp. xyli*	EU293373	TGF15	2 / 2	K	Gram +	6	A+F
70/TGF15	*Mycobacterium anthracenicum*	EU293382	TGF15	2 / 2	K	Gram +	0	A, F, A+F
72/TGF15	*Agrococcus casei*	EU293376	TGF15	3 / 3	K	Gram +	1	A+F
95/TGF15	*Agromyces aurantiacus*	EU293381	TGF15	4 / 4	K	Gram +	3	A+F
F/TGF15	*Coniochaeta ligniaria*	EU450836	TGF15	2 / 2	K	Fungus	6	A, A+F

[a] Lettuce seed germination assay with significant improvement of germination ($P = 0.05$) / total number of assays performed [115]

[b] Toxic compounds: phenol, 2-methoxyphenol, 2,6-dimethoxyphenol, 2-furalaldehyde, pyrrole-2-carboxaldehyde and furan-2-methanol [115]

[c] Growth in mineral salt medium with acetic acid (A) (1%), formic acid (F) (1%) and acetic acid (0.5%) + formic acid (0.5%) (A+F) as sole carbon source [115]

suspension diluted 1000-fold to obtain inoculum corresponding to approximately 10^5 cell number per ml suspension. The densities of the bacterial and fungal inoculants were checked by dilution plating on R2A.

Colonization of TGF with single bacteria and bacterial consortia

The selected bacteria described in Table 1 were tested as single strains for their capacity to colonize TGF in several independent experiments. Survival was determined by plate counts after 3 and 5 days. Bacterial cell densities in TGF were determined by dilution plate counting. For this purpose, 5 ml of 0.85% NaCl solution was added to each TGF microcosm. The treated TGF was shaken for 10 min at max. speed on a shaker (SF1, Stuart Scientific, Great-Britain). Serial tenfold dilutions were prepared in sterile saline and spread-plated in triplicate on R2A. Plates were incubated at 25 ºC. Colonies were counted every 2 days up to 12 days. The counts were then expressed per g dry TGF and transformed to logarithmic scale.

A bacterial consortium consisting of r and K strategists was introduced into TGF. We thus combined selected gram-negative and gram-positive bacteria, namely *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, *S. maltophilia* 34/TGE5, *R. radiobacter* 43/TGE20, *F. denitrificans* 48/TGE20 and *A. aurantiacus* 95/TGF15. Also a consortium containing of six gram-positive bacteria, namely *L. poae* 83/TGF15, *L. xyli subsp. xyli* 93/TGF15, *Afipia broomeae* 94/ TGF15, *L. poae* 97/TGF15, *L. poae* 108/TGF15, and *Afipia broomeae* 109/TGF15 was prepared by combining these cultures as inocula. Inoculation of TGF was done as described above. Survival was determined by assessing the CFU numbers appearing on plates following dilution plating on the relevant media after 1, 24, and 96 h.

Colonization of TGF with combinations of bacteria and C. ligniaria F/TGF15

The bacterial strains *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, *M. radiotolerans* 56/TGF10, *L. xyli subsp. xyli* 66/TGF10, *M. anthracenicum* 70/TGF15, *A. aurantiacus* 95/TGF15, and a mix of *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5 and *M. radiotolerans* 56/TGF10 were combined with *C. ligniaria* F/TGF15 to test their ability to colonize TGF. As a control, *C. ligniaria* F/TGF15 was also introduced to the TGF without inoculant bacteria. Survival of the inoculant bacteria as well as *C. ligniaria* was determined by dilution plate counts on the relevant media after 7 days as described previously.

Effect of nutrients and surfactant on TGF colonization

To test if the colonization of TGF by bacterial strains was affected by a lack of nutrients or by TGF surface specifics, one ml suspension (containing 10^8 cells per ml) of

selected organisms, i.e. (1) *M. radiotolerans* 56/TGF10 or (2) the bacterial consortium with seven strains in roughly equal CFU numbers, was mixed with 0.6 ml AFY solution (0.5% acetic acid + 0.5% formic acid + 0.001% yeast extract in MSM), 0.6 ml surfactant (0.01% or 0.001% Silwet in MSM), or a combination of both. These suspensions were added to the TGF. Survival was determined by dilution plate counts on the relevant media (see above) after 7 days.

Effect of washing and sterilization on TGF colonization

In this experiment, non-sterilized, gamma-irradiated and washed gamma-irradiated TGF samples were used. The washed gamma-irradiated samples were prepared as follows: 19 g TGF was mixed with 500 ml distilled water and stirred for 2 h using a magnetic stirrer at 700 rpm. Grass fibers were collected on filter paper and washed once more in distilled water during 15 min. After collection of the grass fibers on filter paper, they were dried at room temperature in a flow cabinet for 3 days. These steps were all performed under sterile conditions. A bacterial-fungal consortium consisting of *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, *M. radiotolerans* 56/TGF10, *L. xyli subsp. xyli* 66/TGF10, *M. anthracenicum* 70/TGF15, *A. aurantiacus* 95/TGF15 and *C. ligniaria* F/TGF15 was prepared by combining roughly equal CFU numbers (about 10^5 per ml final concentration) of all inocula. In some treatments, additional nutrients were added. Thus, 0.8 ml inoculum was mixed with 0.8 ml AFY (0.5% acetic acid + 0.5% formic acid + 0.001% yeast extract, in MSM medium) before addition to the TGF. After 7 d incubation, survival was determined by dilution plate counting. Bacterial composition was also assessed by direct molecular means, i.e. by PCR-DGGE performed on DNA directly extracted from TGF.

Colonization of TGF after precolonization by C. ligniaria F/TGF15

TGF was inoculated with 1.6 ml of a *C. ligniaria* F/TGF15 suspension in MSM medium containing approximately 10^4 propagules per ml. The matrix was mixed, and incubated at 25 ºC. A bacterial consortium (0.8 ml, containing 10^5 cells per ml) of *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, *M. radiotolerans* 56/TGF10, *L. xyli subsp. xyli* 66/TGF10, *M. anthracenicum* 70/TGF15 and *A. aurantiacus* 95/TGF15 was introduced either 3 h, or 1, 3, 6 and 13 days later. Seven days after inoculation with the consortium, survival of all inoculants was determined by dilution plate counting on R2A medium. Plates were incubated at 25 ^{o}C and checked every 2 up to 12 days. Colonies of *S. plymuthica* 23/TGE5 and *M. radiotolerans* 56/TGF10 were recognizable by colony morphology and these were thus counted separately. This experiment was repeated under exactly the same conditions.

Furthermore, the bacterial composition of the TGF was assessed with PCR-DGGE. Additionally, 25 colonies randomly obtained from plates were identified by partial sequencing of the 16S rRNA [115].

Analysis of bacterial community composition in TGF by PCR-DGGE and colony PCR

The bacterial composition of the inoculated TGF was analyzed with PCR-DGGE as follows. DNA was extracted from TGF using the UltraClean™ soil DNA kit (MoBio Laboratories, BIOzymTC, Landgraaf, the Netherlands), as described [115]. The DNA from the four gram-negative bacteria was isolated as described [115] and DNA from the three gram-positive bacteria was extracted by using the Master Pure Gram Positive DNA Purification Kit (EPICENTRE , USA). Crude DNA was obtained from single colony material and the resultant crude lysates were used as templates for PCR.

Amplification of 16S ribosomal (r)RNA genes was performed by using PCR in 50-µl reaction volumes containing 0.2 µM of each primer, 3.75 mM $MgCl_2$ (Perkin-Elmer, Nieuwersluis, the Netherlands), 200 µM of each dNTP (Boehringer, Almere, the Netherlands) and 0.25 µg of T4 gene 32 protein (Boehringer, Mannheim, Germany) using 5 U Ampli*Taq* Stoffel fragment in Stoffel buffer. The primers used were bacterial 16S rRNA gene forward primer U968 (5'-AACGCGAAGAACCTTAC-3') and reverse primer R1378 (5'-CGGTGTGTACAAGGCCCGGGAACG-3') [44]. For DGGE, a GC-clamped version of U968, i.e. U968-GC (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCTTAC-3') was used. Thermal cycling was as follows: initial denaturation at 94^0 C for 5 min, followed by 35 cycles of 94 ^{0}C - 1 min, 65 ^{0}C – 90 s, 72 ^{0}C – 2 min, final extension at 72 ^{0}C for 10 min. The PCR products (expected sizes about 450 bp) were analyzed by running 5-µl aliquots of the reaction mixtures in 1 % agarose gels.

For DGGE analyses, 6% polyacrylamide gels (ratio of acrylamide to bisacrylamide 37:1) were used with a gradient of 45% to 65% denaturants (100 % denaturant was defined as 7 M urea plus 40% formamide). The gels were run at 60 ºC (100 V) for 16h in a Phor-U2 apparatus (Ingeny International, Goes, the Netherlands) and stained with SYBR gold (Molecular Probes, Leiden, the Netherlands).

PCR products of single colonies were purified with the High-Pure PCR purification kit (Roche GmbH, Mannheim, Germany) and then sequenced using an ABI Prism automatic sequencer (Greenomics, Wageningen, the Netherlands).

Statistical analysis

Analyses of variance (ANOVA) were performed on the log-transformed CFU values with the statistical program Genstat 8 (Rothamsted Experimental Station,

Harpenden, UK). Least significant differences (LSD) were calculated at a significant level of P=0.05.

Results

In vitro antagonism of selected TGF bacteria towards a range of fungi

To create a TGF substrate, which will finally be suppressive against phytopathogenic fungi, in vitro antagonism of the 11 selected bacteria towards 10 fungi, was tested. From the 11 strains tested, three inhibited one or more fungi (Table 2). Two types of inhibition zones were observed, i.e. a complete inhibition of spore germination or hyphal growth, and a growth reduction resulting in retarded growth. The isolate *S. plymuthica* 23/TGE5 inhibited all 10 fungi tested and *P. corrugata* 31/TGE5 inhibited 9 of the fungi tested (except *Penicillium sp*.), whereas isolate *S. maltophilia* 34/TGE5 only partially inhibited two in ten tested fungi (Table 2). The other bacterial isolates did not inhibit any of
the tested fungi. The bacteria and fungi were grouped as to their interrelationships but we did not find any pattern of grouping, nor correlation from the phylogenetic point of view.

Colonization of TGF with single bacteria and bacterial consortia

Following introduction of approximately 10^5 CFU per g matrix, none of the 11 selected bacterial strains survived after 3 or 5 days in TGF, as in all cases their CFU numbers dropped to below detection (estimated at 200 CFU per g). Also, the consortium of the six selected gram-positive bacteria did not survive after 3 and 5 days presence in the matrix (data not shown).
The consortium of the seven selected bacterial strains *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, *M. radiotolerans* 56/TGF10, *L. xyli subsp. xyli* 66/TGF10, *M. anthracenicum* 70/TGF15 and *A. aurantiacus* 95/TGF15 also did not show any persistence as culturable forms in the matrix (3 and 5 days, detection limit 200 CFU per g). A very similar result was found when combined gram-negative and gram-positive bacteria were used, in the consortium consisting of *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, *S. maltophilia* 34/TGE5, *R. radiobacter* 43/TGE20, *F. denitrificans* 48/TGE20 and *A. aurantiacus* 95/TGF15. In this case, measurements taken shortly following the introduction revealed that the population as-a-whole gradually decreased after its introduction at 10^5 CFU per g into the TGF, and after 96 h no CFU were detectable anymore (Fig. 1). The progressive CFU death was consistent across several

experiments. Overall, these data show that the TGF matrix exhibits a generally bactericidal effect on bacterial inoculants.

Table 2 *In vitro* anatgonism of three bacterial isolates that inhibited one or more fungi

Isolate Nr.	Name of closest affiliate	*Fusarium sp.*		*Fusarium oxysporum fsp. radicis lycopersici*		*Fusarium oxysporum fsp. radicis lycopersici*		*Penicillium sp.*		*Pythium ultimum*		*Pythium aphani-dermatum*		*Rhizoctonia solani*		*Rhizopus. sp.*		*Tricho-derma harzianum*		*Verticillium dahliae*	
		1[a]	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
23/TGE5	*S. plymuthica*	+[b]	-	-	+	-	++	+	+++	+	-	+	-	++	-	+	-	+	++	+	++
								+						+							
31/TGE5	*P. corrugata*	-	+	-	+	-	+++	-	-	+	-	-	+	+	+++	+	-	-	+	++	++
										+										+	
34/TGE5	*S. maltophilia*	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-

[a] 1, complete inhibition of fungal spore germination or mycelium growth; 2, partial inhibition, slower growth and germination

[b] Size of inhibition zones: +++, > 10 mm; ++, 5-10 mm; +, 0-4 mm; -, no inhibition zone.

Colonization of TGF with combinations of bacterial strains and C. ligniaria F/TGF15

Table 3 summarizes the bacterial and fungal numbers present in the TGF after 7 days of incubation. Three days after introduction, each of the bacterial strains tested was already below the limit of detection, i.e. log 2 CFU per g dry TGF. Seven days after introduction, in the combination with *C. ligniaria*, none of the bacterial strains was detectable anymore.

Table 3. Colonization of TGF after inoculation with the fungus *Coniochaeta ligniaria* F/TGF15 alone, or in combination with single bacterial isolates or a bacterial mix. Populations are measured after 7 days incubation at 25 °C.

Inoculated isolates	Bacterial inoculum (Log CFU/g dry TGF)	Bacterial population (Log CFU/g dry TGF)	Fungal inoculum (Log CFU/g dry TGF)	Fungal population (Log CFU/g dry TGF)
F/TGF15	-	-	4.3	10.5
15/TGE5 + F/TGF15	5.6	< 2[a]	4.8	10.1
23/TGE5 + F/TGF15	5.6	< 2[a]	4.8	11.2
31/TGE5 + F/TGF15	5.6	< 2[a]	4.3	10.3
56/TGF10 + F/TGF15	5.6	< 2[a]	4.8	9.4
66/TGF10 + F/TGF15	5.6	< 2[a]	4.8	9.7
70/TGF15 + F/TGF15	5.6	< 2[a]	4.3	11.7
95/TGF15 + F/TGF15	5.6	< 2[a]	4.3	12.2
23/TGE5 + 31/TGE5 + 56/TGF10 + F/TGF15	5.6	< 2[a]	4.8	10.2
LSD [b]				0.4

[a] the detection limit of the method is log 2 CFU/g TGF

[b] Least significant difference at $P = 0.05$

In contrast, *C. ligniaria* F/TGF15 revealed growth in the TGF matrix, from approximately log 4.5 per g dry material at the onset of the experiment to over log 9.4 CFU per g dry TGF after 7 days. Similar fungal growth was found in all cases, i.e. upon single introduction or in the combination with any of the bacterial strains.

Colonization of TGF by bacterial strains and C. ligniaria F/TGF15 under varied conditions

Table 4 summarizes the data on the colonization of differently-treated TGF samples. Again, *C. ligniaria* F/TGF15 was able to grow on TGF in all treatments, from log 4.7 CFU per g up to log 8.6 - log 9.9 CFU per g dry TGF. As expected, all bacterial inoculants died out as CFU within 3 days. Prior gamma sterilization of the TGF did not exert any effect on the bacterial colonization, irrespective of the presence of *C. ligniaria*

F/TGF15. However, washing of TGF did enhanced the colonization of TGF with all bacterial inoculants tested: following inoculation with approx. log 5 CFU per g, at the end of the experiment (7 days) 8.4 log CFU were present in sterilized washed TGF, whereas log 7.0 CFU appeared in sterilized washed TGF with *C. ligniaria* F/TGF15 (Table 4). The addition of extra (0.5% acetic acid + 0.5% formic acid + 0.001% yeast extract in MSM) nutrients or the combined application with the fungus did not enhance colonization since the final number of persisting bacteria was reduced (only log 6.4 CFU bacteria).

Table 4. Colonization of differently treated TGF after inoculation with the fungus (F) *Coniochaeta ligniaria* F/TGF15 in combination with a bacterial mix (B mix) of isolates *P. putida* 15/TGE5, *S. plymutica* 23/TGE5, *P. corrugata* 31/TGE5, *M. radiotolerans* 56/TGF10, *L. xyli subsp. xyli* 66/TGF10, *M. anthracenicum* 70/TGF15, and *A. aurantiacus* 95/TGF15. Populations are measured after 7 days incubation at 25 °C.

Treatments of TGF			Microbial inoculum	Bacterial inoculum (Log CFU / g dry TGF)	Bacterial population (Log CFU / g dry TGF)	Fungal inoculum (Log CFU / g dry TGF)	Fungal population (Log CFU / g dry TGF)
-	-	-	B mix + F	5.3	< 2[a]	4.7	8.9
Sterilized	-	-	B mix + F	5.3	< 2[a]	4.7	8.6
Sterilized	Washed	-	B mix	5.3	8.4	ND[b]	ND[b]
Sterilized	Washed	-	B mix + F	5.3	7.0	4.7	9.2
Sterilized	Washed	Nutrients	B mix + F	5.3	6.4	4.7	9.9
LSD [c]					0.3		1.0

[a] Detection limit is log 2 CFU/g TGF

[b] ND - not determined

[c] Least significant difference at $P = 0.05$

Direct analysis of the bacterial community structure in TGF with PCR-DGGE of matrix-extracted DNA (Fig. 1) revealed that all 7 introduced strains in the washed, washed-plus-fungus and washed-plus-fungus-plus-nutrients treatments could be tracked back. Strains *Serratia plymuthica* 23/TGE5 (band 1) and *Pseudomonas putida* 15/TGE5 (band 5) appeared to be relatively dominant. The only exception was strain *Methylobacterium radiotolerans* 56/TGF10, of which the presence could not be proven, since band 7 corresponded also to isolate *Pseudomonas corrugata* 31/TGE5.

Colonization of TGF by a bacterial consortium after pre-colonization with C. ligniaria F/TGF15

Since *C. ligniaria* F/TGF15 was consistently able to colonize TGF, and in the light of its proven ability to break down phytotoxic compounds [115], TGF was pre-colonized

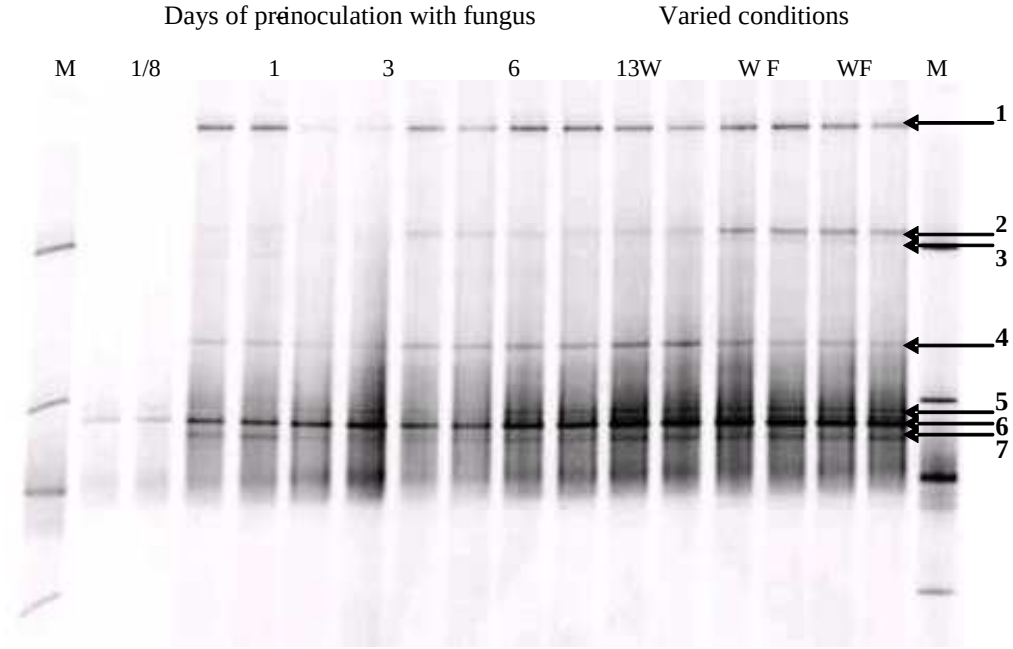

Fig. 1. Fingerprint by PCR-DGGE of the bacterial communities in differently treated TGF samples. TGF was pre-inoculated with the fungus *Coniochaeta ligniaria* F/TGF15 1/8, 1, 3, 6 and 13 days before the bacterial mix was added. TGF samples were washed (W), with or without added nutrients (N) and inoculated together with the fungus (F). The bacterial mix consisted of 15/TGE5, 23/TGE5, 31/TGE5, 56/TGF10, 66/TGF10, 70/TGF15, and 95/TGF15. "M" is a general bacterial marker. The band numbers corresponded with the following strains 1- strain 23/TGE5, 2- strain 66/TGF10, 3 –strain 70/TGF15, 4 –strain 95/TGF15, 5 – strain 15/TGE5, 6- strains 15/TGE5 and 31/TGE5, and 7 - strains 31/TGE5 and 56/TGF10

with this fungus for different periods of time, on the assumption that this pre-growth might also remove the putative bactericidal compounds from the matrix. Thereafter, the consortium of 7 selected bacterial strains was added at log 5.8 CFU/g dry TGF. The results showed that after 3, 6 and 13 days of precolonization with the fungus, the consortium could grow in the TGF, from about log 5.8 up to about log 10 CFU per g TGF (Table 5). Approximately 10 % of this population consisted of *S. plymuthica* 23/TGE5 and/or *M.*

radiotolerans 56/TGF10, as evidenced from the pink colonies appearing on plates (Table 5). On the other hand, precolonization of the TGF by *C. ligniaria* for just 3 h or 1d did not facilitate bacterial growth or persistence, as the CFU counts dropped to below the detection limit, i.e. log 2 cells per g dry TGF.

The 25 random colonies that were checked for their identity by colony PCR, were: *P. putida* 15/TGE5 12 x (48%) of the population, *S. plymuthica* 23TGE5 3 x (12%), *P. corrugata* 31/TGE5 1 x (4%) and *M. radiotolerans* 56/TGF10 5 x (20 %). Hence, at least four species were found to persist, as dominating members of the community that yielded CFU. DNA extraction or PCR from four colonies failed, and these might be the gram-positive strains *L. xyli subsp. xyli* 66/TGF10, *M. anthracenicum* 70/TGF15 and *A. aurantiacus* 95/TGF15. The latter were still present in the community in the TGF matrix, as they were found on DGGE as bands 2, 3 and 4 (Fig. 2).

The direct PCR-DGGE analysis of the bacterial community structures (Fig. 2) showed that *S. plymuthica* 23/TGE5 (band 1), *P. putida* 15/TGE5 and/or *P. corrugata* 31/TGE5 (bands 5, 6 and 7), *L. xyli subsp. xyli* 66/TGF10 (band 2), *M. anthracenicum* 70/TGF15 (band 3) and *A. aurantiacus* 95/TGF15 (band 4) were present in the TGF pre-colonized with *C. ligniaria* F/TGF15. In this analysis, the band of *M. radiotolerans* 56/TGF10 was found to overlap with the band of *P.corrugata* 31/TGE5.

Discussion

Eleven bacterial strains and one fungus, initially isolated from enrichments in TGF or its extract, were selected to actively colonize freshly-prepared TGF, in an attempt to establish a plant-beneficial microbial community on this substrate. As several bacterial strains originated from sequential enrichment cultures in the TGF itself [115], it was expected that these strains would easily colonize the TGF. Unexpectedly though, TGF was found to be very recalcitrant to bacterial colonization. None of the selected bacteria, applied either as single strains or as combinations of strains (consortia), was able to colonize and persist in the matrix.

This analysis included various gram-negative and gram-positive bacteria, the latter including actinobacteria which abounded in the enrichment cultures of TGF [115]. In fact, these bacteria all showed rapid declines of CFUs following their introduction into TGF.

We first hypothesized there might have been a necessity for easily-available nutrients in the colonization process or, alternatively, a problem with a too hydrophobic nature of the fibers. However, addition of selected nutrients or of a surfactant did not enhance the survival of any of the bacterial inoculants in the TGF. We then surmised that TGF might contain bacteriostatic or bacteriocidal factors of unknown nature that could possibly be removed by cold-water washing. Indeed, washing of the TGF allowed several

bacterial inoculants namely *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata*31/TGE5 and *M. radiotolerans* 56/TGF10 to establish and grow on the TGF. Hence, we concluded that TGF contained as-yet-unknown water-soluble compounds that are detrimental to the persistence and growth of the selected bacteria.

Table 5. Colonization of TGF after pre-inoculation with the fungus *Coniochaeta ligniaria* F/TGF15 during 1/8, 1, 3, 6 and 13 days by the bacterial mix of isolates 15/TGE5, 23/TGE5, 31/TGE5, 56/TGF10, 66/TGF15, 70/TGF15, and 95/TGF15. Populations are measured after 7 days incubation at 25 °C in two independent experiments.

Pre-colonization time with fungus (days)	Bacterial inoculum (Log CFU/ g dry TGF)	Bacterial population (Log CFU/ g dry TGF)	Pink bacterial colonies [b] (Log CFU/ g dry TGF)	Fungal inoculum (Log CFU/ g dry TGF)	Fungal population (Log CFU/ g dry TGF)
Experiment 1					
1/8	5.7	< 2[a]	2[a]	4.6	9.1
1	5.7	< 2[a]	2[a]	4.6	10.8
3	5.7	10.5	8.8	4.5	10.1
6	5.7	10.3	9.3	4.3	10.5
13	5.7	10.4	8.6	4.4	10.5
LSD [c]		0.3			0.6
Experiment 2					
1/8	5.9	2[a]	2[a]	3.9	9.1
1	5.9	2[a]	2[a]	3.9	8.9
3	5.9	10.0	9.0	4.7	8.9
6	5.9	9.8	8.9	4.1	9.2
13	5.9	9.8	7.5	4.4	9.1
LSD [c]		0.4			0.6

[a] Detection limit of the method is log 2 CFU/g TGF

[b] Isolates 23/TGE5 and 56/TGF10 both form pink colonies

[c] Least significant difference at $P = 0.05$

In contrast to the bacterial strains, the fungus *Coniochaeta ligniaria* was shown to consistently grow and persist on TGF. This fungus had been isolated from TGF and it thus turned out to be a key element in our further developmental research. Previous research had

already shown the capacity of *C. ligniaria* to break down several phytotoxic compounds that are present in TGF extract, including phenol, 2,6-methoxyphenol and 2-furalaldehyde [115]. *C. ligniaria* is also known to play a role in the biological detoxification of lignocellulosic hydrolysates. [70, 71]

Precolonization of TGF for 3 days or more with *C. ligniaria* was shown to pave the way for subsequent bacterial growth on TGF. The most likely explanation for this effect is that the concentrations of key compounds that inhibited bacterial colonization and growth were strongly reduced by the fungal growth. This hallmark finding provides an indication for the role of *C. ligniaria* as a mediator organism, promoting bacterial growth by removing bacteriostatic or bactericidal compounds from TGF. The resultant strategy of combining fungal and bacterial inoculants to achieve both establishment and persistence of inoculants can have important repercussions in the applied areas of ecology, e.g. in bioremediation. In the environment, *C. ligniaria* has been found on pine sapwood samples [92]. It has also been studied in bioabatement processes as a potential agent for removing inhibitory compounds from lignocellulose hydrolysates [78].

The cultural bacterial community that persisted in the *C. ligniaria* - treated TGF consisted of minimally four strains, i.e. *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5 and *M. radiotolerans* 56/TGF10. All are gram-negative bacteria, three of them being r-strategists and one, *M. radiotolerans* 56/TGF10, a K-strategist. Moreover, strains *L. xyli subsp. xyli* 66/TGF10, *M. anthracenicum* 70/TGF15 and *A. aurantiacus* 95/TGF15 which are all gram-positive bacteria, although not found in the 25 random isolates, were definitely present in the community, as their bands were found in the DGGE banding pattern.

The four gram-negative bacterial strains which persisted in the TGF all had plant health promoting potential. Strain 23/TGE5, identified as a *S. plymuthica*, had *in vitro* antagonism towards several phytopathogenic fungi, i.e. *Rhizoctonia solani*, *Fusarium oxysporum* sp. *radicis lycopersici*, *Verticillium dahliae*, *Pythium aphanidermatum* and *Pythium ultimum.* Species of *Serratia* encompass many biocontrol agents [54, 85, 97, 110] and they are prime producers of the antibiotic pyrrolnitrin [31, 69, 75]. Also strain 31/TGE5, identified as *P. corrugata*, showed *in vitro* antagonism towards all aforementioned phytopathogenic fungi. *Pseudomonas* strains with antagonistic properties against soil-borne pathogens have been studied intensively [18, 30]. *P. corrugata* strains are known as antagonists of *Botrytis cinerea [40]*, *Pythium aphanidermatum* and *Pythium ultimum* [33, 37, 106], *Ralstonia solanacearum biovar* 2 [121], *Fusarium oxysporum* [24, 105], and *Monilinia fructicola* [108]. Although no fungal plant pathogen inhibition was found with the isolates *P. putida* 15/TGE5 and *M. radiotolerans* 56/TGF10, those bacteria are also potential plant-beneficial bacteria [40, 57, 133].

In conclusion, we aimed to valorize the plant growth substrate TGF with a stable microbial consortium consisting of *r* and *K* strategists and with the potential to improve plant health. The initial colonization of TGF by *C. ligniaria* F/TGF15 was found to be crucial to allow establishment of bacterial beneficials. A consortium of seven bacterial strains, four of which well known as plant-health-promoting species, was thus consistently established, at high CFU numbers, in the TGF matrix. This indicates the potential of microbiologically-matured TGF as a new substrate allowing the stable and consistent introduction of a vital plant-health-improving microbial consortium. We are currently testing the effect of the presence of the microbial consortium in the TGF matrix on the growth and health of plants supported by it.

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CHAPTER 5

Interactions of plant-beneficial bacteria with the ascomycete *Coniochaeta ligniaria*

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Abstract

Although bacterial-fungal interactions are progressively being recognized as important in many ecosystems, there is still insufficient knowledge in this area, on the one hand because of the complexity of many interactions and on the other hand because of our poor understanding of the mechanisms involved. The aim of this study was to assess the interactions between the ascomycete *Coniochaeta ligniaria* F/TGF15 obtained from torrefied grass fibers (TGF) and selected gram-negative and gram-positive bacteria from the same substrate. Both the influence of these bacteria on the fungus and the effect of the fungus on the bacteria were investigated. *Pseudomonas putida* 15/TGE5, *Methylobacterium radiotolerans* 56/TGF10, *Serratia plymuthica* 23/TGE5, *Pseudomonas corrugata* 31/TGE5, *Leifsonia xyli subsp. xyli* 66/TGF10, *Mycobacterium anthracenicum* 70/TGF15 and *Agromyces aurantiacus* 95/TGF15 were translocated on the *C. ligniaria* F/TGF15 strain which was grown on potato dextrose agar, but not on the agar medium itself. Moreover, *P. putida*, *P. corrugata*, *L. xyli subsp. xyli* and *A. aurantiacus* strains were able to grow on compounds released by the fungus, reaching high cell densities (log 9.1, 7.9, 8.2 and 7.2 per ml, respectively) from starting inocula of approximately log 3 per ml. *M. radiotolerans*, however did not grow on the fungal compounds. Antagonism towards the fungus was observed for *S. plymuthica* and *P. corrugata*, which killed it. Furthermore, *P. putida* was translocated by the fungus in TGF up to at least 6 cm. It also multiplied on fungal hyphae in TGF, reaching CFU densities of log 8.4 per g dry TGF containing the fungus in 20 d, while the strain could not grow in TGF itself. *M. radiotolerans* was not translocated and did not multiply on the fungal hyphae.

Introduction

Fungi play key roles in complex natural environments, and they may provide ecological opportunities for bacteria that occur in their surroundings (Boer *et al.*, 2005; Kohlmeier *et al.*, 2005; Warmink personal communication). Fungi are key inhabitants of numerous complex organic substrates, in which they find their (varying) niches. Torrefied grass fibers (TGF), which are being proposed as a matrix for potting soil (Trifonova *et al.*, 2008b), represent a microhabitat that can be colonized by fungi, in particular the ascomycete *Coniochaeta ligniaria* F/TGF15. Moreover, TGF colonized by beneficial microbial communities has been proposed to provide a great asset to the health of plants that develop on the resulting potting soil (Trifonova *et al.*, 2008c).

In previous work, a range of bacteria was found to be able to colonize the TGF, however successful colonization only occurred in combination with *Coniochaeta ligniaria* F/TGF15. Moreover, some of these bacteria showed antagonism against a range of plant-

pathogenic fungi (Trifonova *et al.,* 2008a). These bacteria thus showed promise as biocontrol constituents of the potting mixes to be produced. However, so far we do not have any understanding of the nature of the putative interactions between these potential biocontrol bacteria and the supporting fungus *C. ligniaria* F/TGF15.

Bacteria that occur in the same microhabitat as particular fungi may show differential behavior in the face of the fungal component. Often, such bacteria may take profit of the presence of the fungus, as the latter organism may provide novel ecological (growth and colonization) opportunities for them. For instance, soil bacteria can benefit from the presence of fungal hyphae, as a result of the presence of fungal-released compounds, such as trehalose, oxalate and mannitol, which may serve as nutrient sources (Andrade *et al.,* 1997; Nurmiaho-Lassila *et al.,* 1997; Zarnowski *et al.,* 2002). The bacteria that associate with fungi may even obtain their benefit at the expense of the fungus, e.g. via predatory action, called mycophagy (De Boer *et al.,* 2007; Hoppener-Ogawa *et al.,* 2007; Johansson *et al.,* 2004). Thus, the chitinolytic bacterium *Collimonas fungivorans* can live by consuming the hyphal tips of *Fusarium spp* (De Boer *et al.,* 2004). It was found that type-III secretion systems, known to facilitate the interaction of both plant and animal pathogens with their hosts, may also play a role in the interaction of bacteria with fungi, in this case the basiodiomycetous ectomycorrhizal fungus *Laccaria proxima*, thus pointing to a role of this bacterial "attack" system in the (nutritional) interaction (Warmink personal communication). Furthermore, many examples of bacterial antagonism against particular fungi are known, i.e. *Paenibacillus polymyxa* being antagonistic against *Fusarium oxysporum (*Dijksterhuis *et al., 1999) Pseudomonas* and *Serratia* spp. against *Verticillium dahliae* (Berg *et al.,* 2006) and *Pseudomonas* spp. against *Rhizoctonia solani* AG3 (Garbeva *et al.,* 2008). Such antagonistic interactions may relate to bacterial-fungal nutrient competition, which is key in the ecology of bacterial and fungal decomposers in aquatic environments (Mille-Lindblom *et al.,* 2006) as well as generally in the food web (Rousk *et al.,* 2008). Where amensalistic behavior may result in complex interactions between populations (Salles *et al.,* 2006; Tanaka *et al.,* 1999), commensalistic behavior is involved in multitrophic interactions between prokaryotes and unicellular eukaryotes (McCreadie *et al.,* 2005). Conversely, fungi involved in the interactions may have evolved metabolic capabilities that allow them to take advantage of the bacteria with which they share the habitat (Van Elsas *et al.,* 2007). For instance, mutualism between fungal and bacterial partners through symbiont-dependent sporulation has been observed by Partida-Martinez *et al* (Partida-Martinez *et al.,* 2007).

TGF is promising for use as a potting soil constituent. Following torrefaction, the material is microbiologically "virgin", allowing the directed establishment of a plant-beneficial microbial community. As, next to particular bacteria, the fungus *Coniochaeta ligniaria* finds its niche in TGF, we were interested in the nature of the putative interactions

between the fungus and these bacteria. In this study, we particularly address the possible inhibitory or stimulatory effects of selected bacteria in the interaction with the fungus.

Material and methods

Microbial isolates

Seven bacterial strains that had been previously selected as candidates for use in the directed colonization of TGF (Trifonova *et al.*, 2008a) were tested in respect of their interactions with *Coniochaeta ligniaria* F/TGF15. These were: *Pseudomonas putida* 15/TGE5, *Serratia plymuthica* 23/TGE5, *Pseudomonas corrugata* 31/TGE5, *Methylobacterium radiotolerans* 56/TGF10, *Leifsonia xyli subsp. xyli* 66/TGF10, *Mycobacterium anthracenicum* 70/TGF15 and *Agromyces aurantiacus* 95/TGF15. Each strain was grown aerobically on R2A plates (Difco, Detroit, MI, USA) at 25 ^{o}C. Long-term storage was in glycerol stocks at -80 °C.

Coniochaeta ligniaria F/TGF15 was grown on potato dextrose agar (PDA; 39 g l^{-1}, Oxoid, London, UK) at 20 ^{o}C in the dark.

Fungal-bacterial interactions on agar medium

Coniochaeta ligniaria F/TGF15 was grown on PDA plate for 10 days, establishing a complete mycelial mat. Then, freshly grown cells of each of the seven bacterial strains were introduced separately by scratching the fungal hyphae with a cell-filled needle half of the Petri dish. Thus, bacteria were spread over the fungal tissue on half of the dish, and the other half remained without added bacteria. The plates were incubated at 25 ^{o}C. All experiments were performed in duplicate. Experimental controls consisted of the seven bacterial strains added on parallel PDA and R2A (Difco, Detroit, MI, USA) agar plates without *Coniochaeta ligniaria* F/TGF15.

Twenty days following introduction, samples were taken from the bacteria-inoculated parts and uninoculated parts of the Petri dishes containing *C. ligniaria* as well as the non-fungal plates. These samples were assessed for bacterial presence and fungal survival. Samples from the uninoculated parts of the Petri dishes were taken distantly from the inoculated part. Using a needle, the tops of aerial hyphae were sampled and samples plated in dilution onto R2A (and PDA) plates supplemented with delvocid (100 mg l^{-1}). Plates were incubated at 25 ^{o}C for 10 days. To assess the fungal response to novel substrate, agar blocks (5 x 5 mm) from the non-bacteria-inoculated parts and from bacteria-inoculated parts were transferred to plates containing R2A and PDA supplemented with chloramphenicol (100 mg l^{-1}). These plates were incubated at 20 ^{o}C and after 15 and 30 days the diameters of the colonies surrounding the agar pieces were measured and the

presence of fungal spores checked by microscopy. Color and morphology of the colonies were also assessed.

Bacterial growth on fungal exudates

Five bacterial strains, i.e. *Pseudomonas putida* 15/TGE5, *Pseudomonas corrugata* 31/TGE5, *Methylobacterium radiotolerans* 56/TGF10, *Leifsonia xyli subsp. xyli* 66/TGF10 and *Agromyces aurantiacus* 95/TGF15, were selected on the basis of their inability to grow on 1% acetic acid over six days (Trifonova *et al.*, 2008b). Cultures in R2A medium were prepared from single colonies. Petri dishes were supplemented with five ml MSM medium (K_2HPO_4 1.4 g l^{-1}, KH_2PO_4 1.4 g l^{-1} , $MgSO_4.7H_2O$ 0.1 g l^{-1}, $(NH_4)_2SO_4$ 1 g l^{-1}, $MnSO_4.7H_2O$ 0.001 g l^{-1}, $FeSO_4.7H_2O$ 0.01 g l^{-1}) containing the suspended single bacterial colonies homogenized with a spatula. The resulting cell suspensions were then transferred to sterile vessels and the optical densities (OD_{600}) adjusted with MSM medium to 0.1 using a BECKMAN DU® 530 Life Science UV/Vis spectrophotometer. Suspensions were then diluted to densities of approximately 10^4 CFU ml^{-1} (checked by plate counting).

Fungal exudates were prepared by growing *C. ligniaria* F/TGF15 in MSM medium containing 1% acetic acid as the sole C source. After incubation for seven days at 20 ^oC and 160 rpm, fungal biomass (mycelium and spores) was removed by centrifugation (9,400 x *g*, 10 min) followed by filter-sterilization of the supernatants (Millex GV bacterial filter Unit 0.22 µm, Millipore). The resulting filtrate containing compounds released by the fungus (fungal exudates) was used as the nutrient solution for growth of the above-described five bacteria. Three ml filtrate was thus mixed with 0.075 ml bacterial inoculum, corresponding to a final concentration of 10^2 cells ml^{-1}. For each bacterial strain, a control consisting of MSM medium supplemented with 1% acetic acid was prepared. All experiments were performed in duplicate. Bacterial CFU in all treatments were determined after one and four days of incubation at 25 ^oC . To obtain CFU counts, serial dilutions prepared in 0.85% NaCl were plated on R2A plates, which were subsequently incubated at 25 ^oC and examined for colony development after 1, 2, 4, 6, 8, up to 10 days.

Translocation of bacteria by fungal hyphae grown on TGF

To assess the translocation of bacteria by *C. ligniaria*, the system developed by Warmink and Boersma (personal communication) was used. The system was slightly modified for our purposes and consisted of Petri dishes with three compartments. One compartment was filled with PDA. The second and third compartments were filled with 2 g dry TGF each, moistened with 6.5 ml MSM medium. TGF was prepared as described (Trifonova *et al.*, 2008b). At the edge of the PDA compartment, agar blocks with *C. ligniaria* were placed. The systems were incubated at 20 ^oC in the dark, which allowed fungal growth. When the fungal hyphae had colonized about 5 mm of the TGF, suspensions

containing 10^4 ml^{-1} cells of *P. putida* 15/TGE5 or *M. radiotolerans* 56/TGF10 were introduced at the hyphal front. These strains had been selected by the criterion of the lack of inhibition of *C. ligniaria* F/TGF15. Each strain was added to two TGF-containing compartments. The systems were incubated at 20 ^oC in the dark. Three h following incubation, fungal hyphae were sampled (duplicate per compartment) and analyzed to determine the bacterial CFU numbers on R2A. Twenty days later - when the fungal hyphae had grown about 60 mm, fungal hyphae together with TGF(0.05g) were sampled in the two compartments at distance of 30 and 60 mm, in duplicate. One ml of 0.85% NaCl solution was added to each sample and tubes were shaken for 10 min at maximum speed (Flask shaker SF1, Stuart Scientific, England). After serial dilution, samples were plated on R2A and incubated for up to 10 days at 25 ºC. CFU were enumerated every two days. Results are presented as log CFU g^{-1} dry TGF. The following controls were included in the experiment: (1) a negative control, which consisted of the fungus grown on agar or TGF without any inoculated bacteria, (2) *P. putida* 15/TGE5 and *M. radiotolerans* 56/TGF10 inoculated onto TGF without the presence of fungal hyphae.

Statistical analysis

Analyses of variance (ANOVA) were performed on log-transformed CFU values with the statistical program Genstat 10 (Rothamsted Experimental Station, Harpenden, UK). Least significant differences (LSD) were calculated at a significance level of P=0.05. Standard deviations were calculated for the CFU data of fungal exudates.

Results

Fungal-bacterial interactions on agar medium

Migration - Table 1 shows the data on the interactions of the seven selected strains with *C. ligniaria* F/TGF15 on PDA plates. In the presence of the fungus, all seven strains were detected at both sides of the Petri dishes, i.e. the inoculated and uninoculated parts. In contrast, in the control PDA and R2A agar plates without *C. ligniaria* F/TGF15, all seven bacterial strains showed growth at the inoculation spot, but none was shown to migrate. All strains thus migrated on PDA that had been colonized by *C. ligniaria* F/TGF15, possibly spreading along the fungal tissue, being transported to the other side of the Petri dish.

Inhibition - Strains *L. xyli subsp. xyli* 66/TGF10, *M. anthracenicum* 70/TGF15 and *A. aurantiacus* 95/TGF15 showed no inhibition of growth of the fungus on PDA blocks containing fungal/bacterial mixes. When introduced together, *S. plymuthica* 23/TGE5 was found to completely inhibit the outgrowth of *C. ligniaria* F/TGF15 on PDA medium. On the other hand, *P. putida* 15/TGE5, *P. corrugata* 31/TGE5 and *M. radiotolerans* 56/TGF10

partially inhibited *C. ligniaria*; fungal growth was 0.3, 0.2 and 0.4 mm/day respectively, versus a regular 0.6 mm/ day in the control without bacteria.

Physiological effects - Fungal hyphal appearance clearly changed, in terms of color and amount of aerial mycelium, due to the interaction with several of the bacterial strains (Table 1). Fungal outgrowth on PDA, in the absence of bacteria (the control), yielded growth 0.6 mm/day, orange-coloured mycelial colonies with purplish brown tinges in the centres and sporulation. The changes in morphology of the *C. ligniaria* F/TGF15 hyphal mats due to interactions with different bacteria are shown in Fig 1. In each combination, there were clear changes in the colors as well as morphologies of the fungal hyphal colonies.

Bacterial growth on fungal-released compounds

Growth of *P. putida* 15/TGE5, *P. corrugata* 31/TGE5, *M. radiotolerans* 56/TGF10, *L. xyli subsp. xyli* 66/TGF10 and *A. aurantiacus* 95/TGF15 in liquid medium (1% acetic acid) with and without exudates released by *C. ligniaria* is presented in Fig. 2. These strains were selected for their inability to grow, for six days, on acetic acid (Trifonova *et al.*, 2008b). In the control medium without fungal-released compounds, *P. corrugata* 31/TGE5, *M. radiotolerans* 56/TGF10 and *L. xyli subsp. xyli* 66/TGF10 did not show any growth, whereas *P. putida* 15/TGE5 and *A. aurantiacus* 95/TGF15 showed slight increases of numbers, from log 3 CFU ml^{-1} to log 5 CFU ml^{-1} and from log 2.2 CFU ml^{-1} to log 3.9 CFU ml^{-1}, respectively.

For all strains except one (*M. radiotolerans* 56/TGF10), significant growth took place over four days in the presence of fungal-released compounds (Fig 2). Thus, all strains except *M. radiotolerans* 56/TGF10 increased their CFU numbers from about log 2.5-3 CFU ml^{-1} to log 7-9 CFU ml^{-1} after 4 days.

Given the positive, respectively negative, growth effect of *C. ligniaria* F/TGF15 on specific bacteria, we selected *P. putida* 15/TGE5 and *M. radiotolerans* 56/TGF10 as model strains for the investigation of possible bacterial translocation via the fungus *C. ligniaria* in TGF.

Colonization of TGF by bacteria in the presence of C. ligniaria F/TGF15

In TGF without *C. ligniaria*, the two selected bacterial strains, *P. putida* 15/TGE5 and *M. radiotolerans* 56/TGF10 (starting inoculum was about 10^{-4} g^{-1} TGF), were not able to survive and get translocated in a period of over 20 d (Table 2). In fact, the bacterial counts at day 20 in these controls at 30 and 60 mm distance from the inoculation point were

Table 1
Growth and morphology of fungus *C. ligniaria* F/TGF15 in the presence of seven selected bacteria and bacterial trans-location by *C. ligniaria* grown on PDA

Bacterial inoculant		Location **inoculated with bacteria**					Location not inoculated with bacteria
Isolate Nr	Affiliated name	Fungal growth (mm/day)	Fungal colony morphology	Status of fungus	Presence of spores	Bacterial presence	Bacterial presence
15/TGE5	*Pseudomonas putida*	0.3	White-pink-yellow, normal mycelium	living	+	+	+
23/TGE5	*Serratia plymutica*	0.0	No mycelium	dead*	-	+	+
31/TGE5	*Pseudomonas corrugata*	0.2	little mycelium	living	+	+	+
56/TGF10	*Methylobacterium radiotolerans*	0.4	pink-orange, normal mycelium	living	+	+	+
66/TGF10	*Leifsonia xyli subsp. xyli*	0.6	Colorless, thinner mycelium than control	living	+	+	+
70/TGF15	*Mycobacterium anthracenicum*	0.6	orange, mycelium with bacterial slime	living	+	+	+
95/TGF15	*Agromyces aurantiacus*	0.6	orange, less mycelium than control	living	+	+	+
control	no bacteria	0.6	Orange	living	+	-	-

* no growth on medium with chloramphenicol (100mg l^{-1})

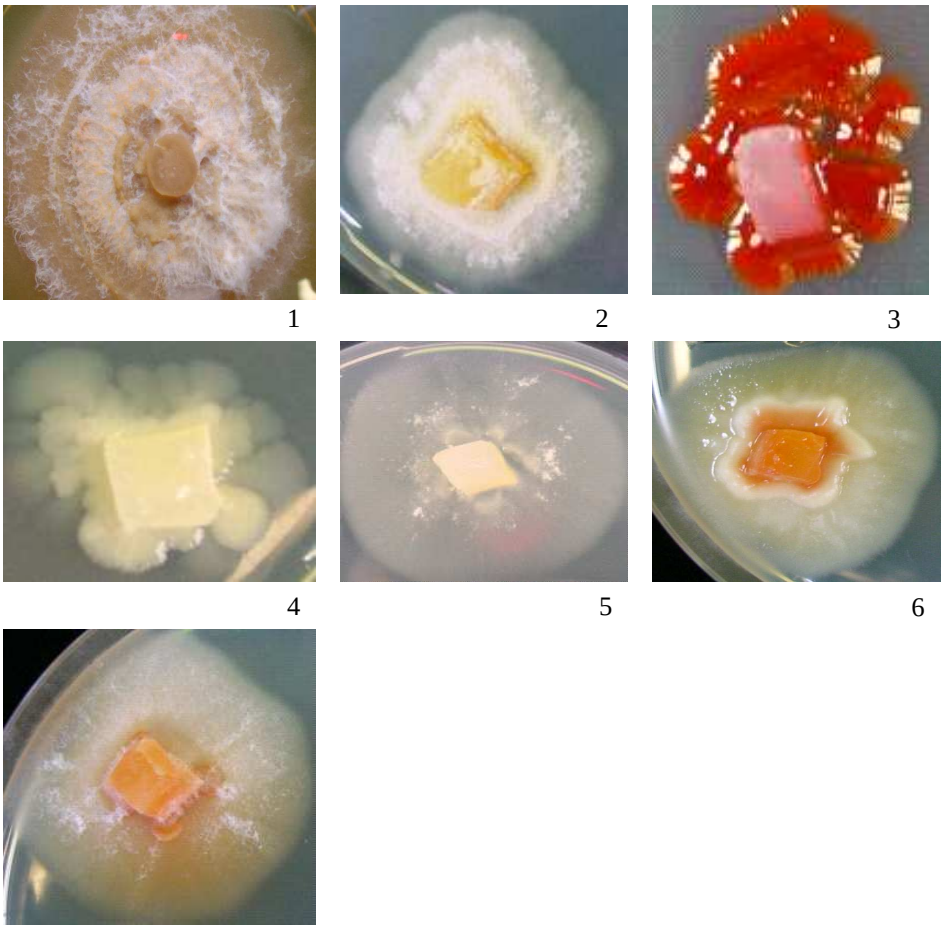
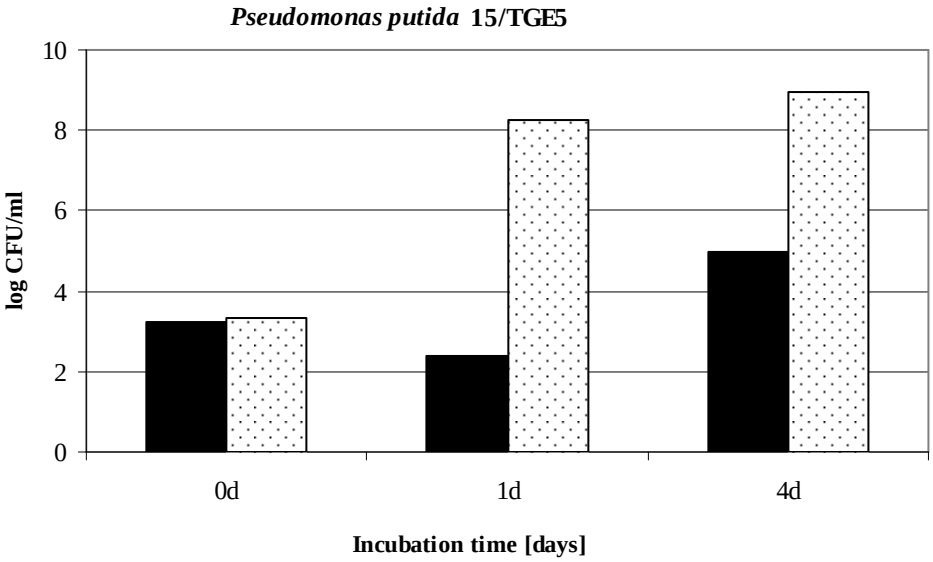
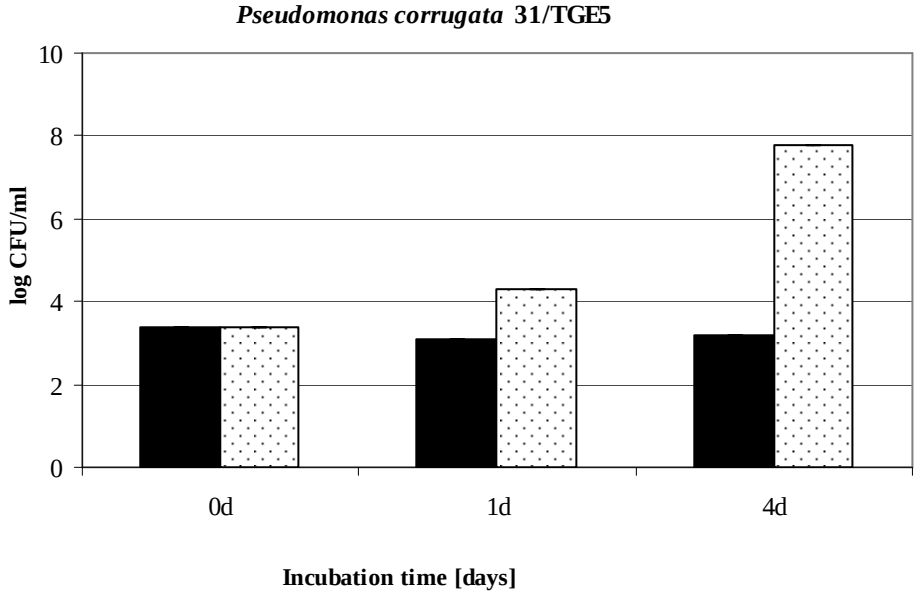
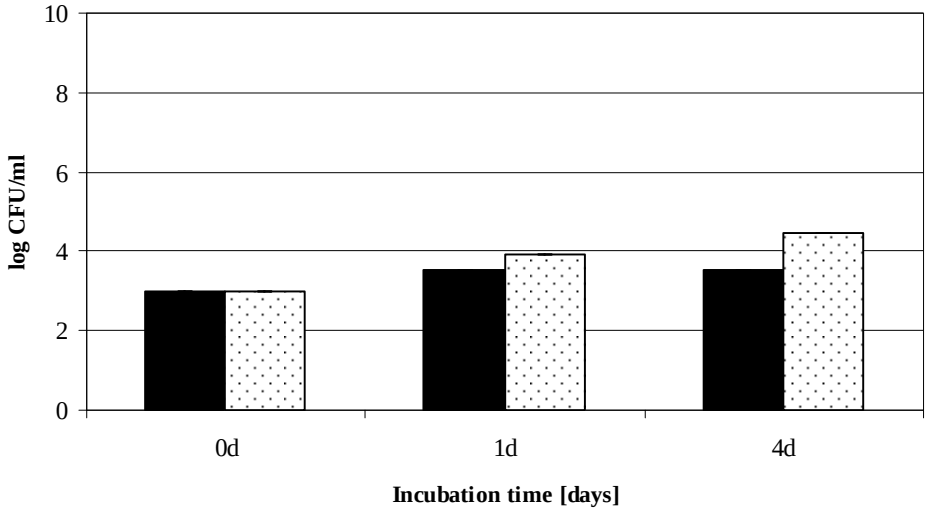


Fig. 1 *Coniochaeta ligniaria* F/TGF15 during outgrowth from a PDA agar plug in the presence of different bacteria; a control without bacteria (1), mycelium in the vicinity of *Pseudomonas putida* 15/TGE5 (2), *Serratia plymuthica* 23/TGE5 (3), *Pseudomonas corrugata* (4) 31/TGE5, *Leifsonia xyli subsp. xyli* 66/TGF10 (5), *Mycobacterium. anthracenicum* 70/TGF15 (6) and *Agromyces aurantiacus* 95/TGF15 (7)

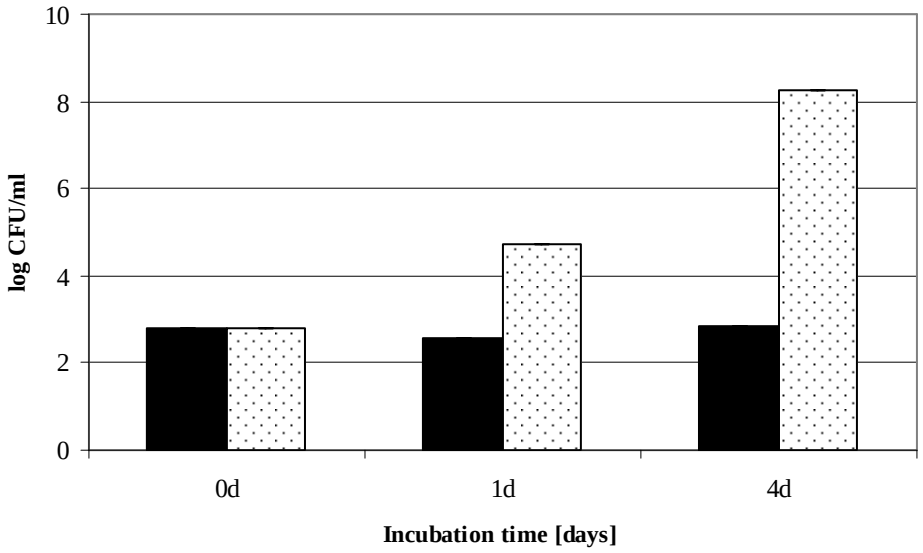

Pseudomonas putida 15/TGE5
log CFU/ml
10
8
6
4
2
0
0d
1d
4d
Incubation time [days]


Pseudomonas corrugata 31/TGE5
log CFU/ml
10
8
6
4
2
0
0d
1d
4d
Incubation time [days]

***Methylobacterium radiotolerans* 56/TGF10**



***Leifsonia xyli subsp. xyli* 66/TGF15**



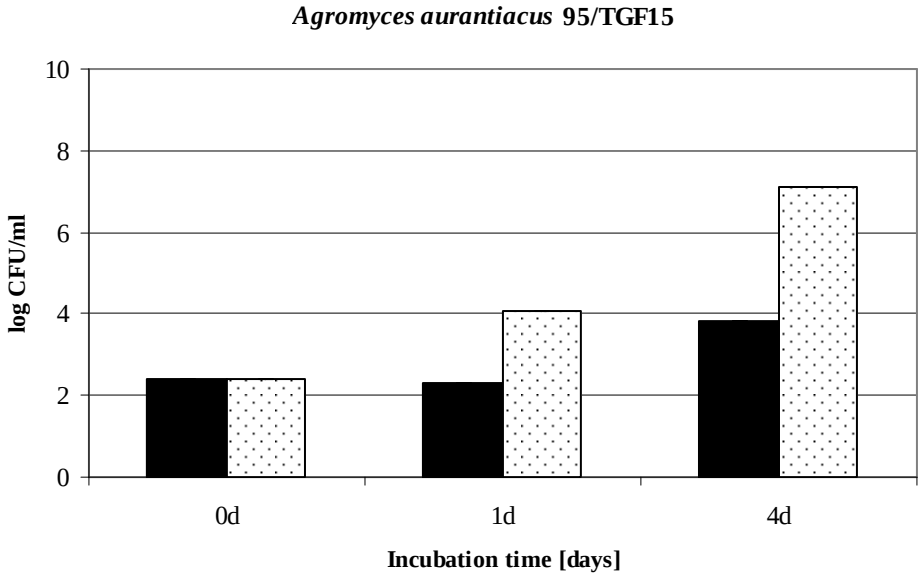


Fig 2 Growth of bacterial strains *P. putida* 15/TGE5, *P. corrugata* 31/TGE5, *M. radiotolerans 56/TGF10*, *L. xyli subsp. xyli* 66/TGF10 and *A. aurantiacus* 95/TGF15 on 1 % acetic acid without exudates (black bars) or with exudates of *Coniochaeta ligniaria* F/TGF15 (striped bars) after 0, 1 and 4 days of incubation at 25 ^{o}C. Least significant difference at P=0,05 is 1.1 (n=2)

below the detection limit of roughly 0.6 log CFU g^{-1} dry TGF. Table 2 shows the migratory behavior of the two strains in the TGF system with *C. ligniaria* F/TGF15. The starting CFU level of *P. putida* 15/TGE5 was about log 4.8 per g dry TGF and that of *M. radiotolerans* 56/TGF10 about log 4.5 per g dry TGF. After 20 d, *P. putida* 15/TGE5 was detected at numbers up to log 8.4 CFU per g dry TGF at distances of 30 and 60 mm from the point of inoculation. However, CFU counts of *M. radiotolerans* 56/TGF10 at these distances were very low (log 1.2 and 0.9 per g dry TGF).

Fungal growth on TGF in the presence of the two bacteria was not significantly different from that without any added bacteria. Thus, the fungal population was 8.3 to 8.9 CFU per g dry TGF irrespective of the presence of added bacteria (Table 3).

Table 2. Colonization of *P. putida* 15/TGE5 and *M. radiotolerans* 56/TGF10 on *C. ligniaria* F/TGF15 grown on TGF (values in log CFU g^{-1} dry TGF) (n=4)

Bacterial inoculant			3 hours	20 days	20 days
Isolate Nr	Affiliated name	Presence or absence of fungus on TGF	Distance [a] 0.5 cm	Distance 3 cm	Distance 6 cm
15/TGE5	*P.putida*	F/TGF15	4.8	8.1	8.4
15/TGE5	*P.putida*	-	4.4	BD [b]	BD [b]
56/TGF10	*M. radiotolerans*	F/TGF15	4.5	1.2 [c]	0.9
56/TGF10	*M. radiotolerans*	-	3.9	BD [b]	BD [b]

[a] Distance to bacterial inoculation point

[b] BD = below detection limit, 0.6 log CFU g^{-1} dry TGF

[c] Mean value of 1 positive value and 3 values below detection limit

LSD = 0.4; Mean values below detection limit were excluded from analysis of variance

Table 3 Growth of fungus *Coniochaeta ligniaria* F/TGF15 on TGF inoculated with the bacterial strains *Pseudomonas putida* 15/TGE5 and *Methylobacterium radiotolerans* 56/TGF10, as well as without bacteria (values in log CFU g^{-1} dry TGF) (n=4)

		3 hours	20 days	20 days
Bacterial inoculant	Name	Distance [a] 0.5 cm	Distance 3 cm	Distance 6 cm
15/TGE5	*P.putida*	8.3	8.4	8.3
56/TGF10	*M. radiotolerans*	7.8	8.3	8.4
Control	No bacteria	8.3	8.9	8.9

[a] Distance to bacterial inoculation point

LSD = 0.6

Discussion

This study investigated the interactions between selected bacteria and the fungal strain *C. ligniaria* F/TGF15 in the context of the colonization of torrefied grass fibers (TGF) by these combined organisms. The organisms had all been isolated from TGF or its extract. The final aim of this study was the establishment of a microbial consortium to be applied in the microbial maturation of TGF for use in potting soil. Several questions were

addressed in the work: a) can the fungus *C. ligniaria* promote bacterial translocation along hyphae, thus promoting bacterial colonization of TGF, b) can any of the bacterial strains utilize compounds that are released by the fungus, c) can any of the bacterial strains multiply on the fungus, d) is the fungus influenced in any way by the bacterial inoculants?

Strikingly, all seven bacteria tested on PDA agar, on which *C. ligniaria* had been grown before, migrated to sites at about 45 mm distance from the inoculation spots, whereas on control plates (without fungus) these strains formed distinct colonies and did not translocate. Movement may have taken place by bacterial growth and/or migration along the hyphae.

In soil, filamentous fungi can favor the distribution of bacteria by providing continuous surfaces and channels on or within which bacteria can move (Kohlmeier *et al.,* 2005). In our study, the bacteria may have used this mechanism to change their location. Fungi may serve as vectors for bacterial transport in the subsurface, and the physical affinity of bacteria to fungal hyphae may mobilize bacteria upon fungal growth ("fungal subway"). Alternatively, continuous water films developing along fungal hyphae might facilitate the displacement of motile bacteria in unsaturated soil ("fungal highway"). Wick *et al.* (2007) found that in particular hydrophilic fungal mycelia bridged air-filled pores and thereby provided a continuous network of waterpaths that enabled bacteria to spread in soil. *C. ligniaria* F/TGF15, being a fungus with hydrophilic cell envelope properties, might have used a similar mechanism facilitating bacterial translocation. Some of our strains may thus have used twitching motility or gliding movement over the hydrophilic surface of *C. ligniaria.*

Furthermore, we tested the possibility that the bacterial strains *P. putida* 15/TGE5, *P. corrugata* 31/TGE5, *M. radiotolerans* 56/TGF10, *L. xyli subsp. xyli* 66/TGF10 and *A. aurantiacus* 95/TGF15 could grow on exudates provided by *C. ligniaria*. To assess bacterial growth, we produced a so-called fungal exudate medium consisting of spent fungal growth medium, where acetate had served as the sole carbon source for *C. ligniaria*. Acetate was known to be a poor carbon source for the five heterotrophic bacteria tested. In fact, no substantial growth was observed for these over five days (Trifonova *et al.,* 2008b). In contrast, in the *C. ligniaria* F/TGF15 exudate medium, all strains except *M. radiotolerans* 56/TGF10 revealed considerable growth over time. Hence, during their translocation on TGF, these bacteria might have been using nutrients released by the *C. ligniaria* host.

Two bacterial strains, *P. putida* 15/TGE5 and *M. radiotolerans* 56/TGF10, were then chosen as the model strains for the investigation of possible bacterial translocation via the fungus *C. ligniaria* in TGF. Translocation of *P. putida* 15/TGE5 was clearly mediated by *C. ligniaria* F/TGF15 on TGF, which was not the case for *M. radiotolerans* 56/TGF10. During a period of 20 days, *P. putida* 15/TGE5 grew in the TGF system in the vicinity of

the fungus and reached log 8.8 CFU per g dry TGF at distances 30 and 60 mm from the inoculation spot. Thus, in the light of the absence of this organism on TGF without added *C. ligniaria*, it is likely that the fungus incited substantial bacterial movement and concomitant growth in the substrate. Although *P. putida* cells were introduced behind the fungal tips, the bacteria might have grown on the fungus and moved together with the growing fungal hyphae. Key to this translocation might be the growth on the fungus. *P. putida* can grow very fast on fungal exudates; in only 24 h reaching 10^8 CFU ml^{-1} from an inoculum of 10^3. In contrast to this, *M. radiotolerans* 56/TGF10 could not migrate with the fungus. Likely, *M. radiotolerans* 56/TGF10 cannot multiply on fungal hyphae, due to its inability to grow in fungal exudates, as shown in a liquid culture. It is known that the expression of flagellar genes in *Methylobacterium* spp. can be dependent on the environmental conditions (Schauer and Kutschera 2008), as cells of sedentary colonies of *M. extorquens* and *Methylobacterium* sp. revealed no sign of flagellum formation when a three-dimensional web of cells was reconstructed. However, bacteria in liquid culture were characterized by a single flagellum. In our study, translocation of *M. radiotolerans* 56/TGF10 occurred on agar medium with the fungus, but not on solid TGF even not when TGF was colonized by the fungus.

In conclusion, different types of interaction were detected between the selected fungus and bacteria in the current experiments. There was no positive influence of the bacteria on the fungus; i.e. fungal growth in TGF was independent on the presence of bacteria. However, a distinct negative or neutral influence of several bacteria on the fungus was detected; i.e. a slower growth, change in color and colony morphology, or death of the fungus in the vicinity of bacteria on an agar medium. The fungus, on the other hand, was able to stimulate the bacterial population. It translocated and promoted growth of *P. putida* 15/TGE5 with the ability to grow on fungal exudates, but not *M. radiotolerans* 56/TGF10 which was not able to grow on the fungal exudates. These results indicate that the fungal exudates probably play a crucial role in the translocation of the bacteria in TGF, facilitating growth of the bacteria along the hyphae. The fact that the fungus caused translocation of all bacteria on an agar medium can be due to the relatively rich en moist environment present in a Petri dish with a nutrient agar.

Four of the seven bacterial isolates tested in this study (namely *Pseudomonas putida* 15/TGE5, *Serratia plymuthica* 23/TGE5, *Pseudomonas corrugata* 31/TGE5 and *Methylobacterium radiotolerans* 56/TGF10, are well known for their plant-growth promoting or antagonistic properties against plant pathogens (Guo *et al.*, 2007; Kai *et al.*, 2007; Trifonova *et al.*, 2008a; Zarnowski *et al.*, 2002). Hence, these organisms are very promising for use in TGF / potting mix applications (Trifonova *et al.*, 2008a). The capacity of such antagonistic bacteria to colonize TGF and migrate in it can enhance their role as plant-health beneficial microorganisms. Hence, our study is important for practical

applications, showing a dual role for *C. ligniaria:* a crucial factor for the colonization and detoxification of TGF, meanwhile stimulating growth and translocation of a consortium of plant-growth-promoting bacteria.

Acknowledgements

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CHAPTER 6

Plant growth on microbiologically enriched torrefied grass fiber - a novel ingredient of potting soil

Trifonova R., Postma J., Schilder M.T. and van Elsas J.D.

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Abstract

The quality of torrefied grass fibers (TGF) as a new potting soil ingredient was tested in a greenhouse experiment. TGF was colonized with previously selected microorganisms. Four colonization treatments were compared: (i) no inoculants, (ii) the fungus *Coniochaeta ligniaria* F/TGF15 alone, (iii) the fungus followed by inoculation with two and (iv) the fungus with seven selected bacteria. Cultivation-based and DNA-based methods, i.e. PCR-DGGE and BOX-PCR, were applied to assess the bacterial and fungal communities established in the TGF. Although colonization was not performed under sterile conditions, all inoculated strains were recovered from TGF up to 40 days incubation. Stable fungal and bacterial populations, of 10^8 and 10^9 CFU/g TGF, respectively, were reached. As a side effect of the torrefaction process that aimed at the chemical stabilization of grass fibers, potentially phytotoxic compounds were generated. These phytotoxic compounds were cold-extracted from the fibers and analyzed by gas chromatography (GC)-mass spectometry (MS). Four of 15 target compounds that had previously been found in the extract of TGF were encountered, namely phenol, 2-methoxyphenol, 2-one-benzopyran, and tetrahydro-5,6,7,7a-benzofuranone. The concentration of these compounds decreased significantly during incubation.

The colonized TGF was mixed with peat (P) in a range of 100%:0% (w/w), 50%:50%, 20%:80% and 0%:100% TGF:P, respectively, to assess suitability for plant growth. Germination of tomato seeds was assessed twice, i.e. with TGF that had been colonized during 12 and 26 days. In both tests, 90-100 % of the seeds germinated in 50%:50% and 20%:80% TGF:P, whereas only 30-60 % of the seeds germinated in pure TGF. Germination was not clearly improved by the microbial inoculants. However, plant weight as well as leaf area of 28-d old tomato plants were significantly increased in all treatments where *C. ligniaria* F/TGF15 was inoculated compared to the control treatment without microbial inoculants. Colonization with *C. ligniaria* also protected the substrate from uncontrolled colonization by other fungi. The added bacteria had a positive effect in few occasions: in the higher TGF concentrations the mix of two bacteria with the fungus showed the highest seed germination, plant weight and leaf area.

Considering plant weight and leaf area, the best option for practical application is the mixture with 20 or 50% TGF colonized with *C. ligniaria* F/TGF15.

Introduction

Thermally-treated (torrefied) grass fibers (TGF) are a viable substitute for peat in potting media [115]. TGF have good water holding capacity and grass is a renewable substrate. Torrefaction was applied in order to transform grass fibers into a (chemically) more stable product [115]. After torrefaction, TGF contains abundant acetic acid [12, 19, 35, 107, 129] as well as formic acid [12, 35] which can serve as nutrient and energy sources for particular microorganisms. Phytotoxic compounds such as phenol, phenolic compounds [83, 114, 115, 122] and 2-furalaldehyde (furfural) [36, 41, 70]are produced as side compounds during the heating process, but they may serve as nutrient sources for specific microorganisms.

A proper microbial colonization of TGF, which is sterile after the heating process, is crucial to obtain a stable microbial community and reduce the phytotoxicity of the substrate. Meanwhile, this is a perfect opportunity to inoculate the substrate with plant-beneficial micro-organisms. Agricultural soil, compost as well as potting soil has often been inoculated with plant-beneficial bacteria or fungi to control plant diseases. The survival of such inoculated organisms has generally been found to be low; mostly less than 1 % survival was noted following incubation [11, 60]. Survival of inoculants in soil has been poor [3, 36, 55]. Also colonization of compost by beneficial microorganisms was often disappointing [67, 90]. A main factor is the lack of competitiveness of the introduced microorganisms versus the indigenous microflora. In the case of TGF, the introduced microorganisms have several advantages: there is no indigenous microflora yet and bacteria and fungi with the ability to grow on the specific (toxic) compounds present in TGF will be in favour.

In our previous work, it was found that the ascomycete *Coniochaeta ligniaria* F/TGF15 together with the bacterial strains *Pseudomonas putida* 15/TGE5, *Serratia plymuthica* 23/TGE5, *Pseudomonas corrugata* 31/TGE5, *Methylobacterium radiotolerans* 56/TGF10, *Leifsonia xyli subsp. xyli* 66/TGF10, *Mycobacterium anthracenicum* 70/TGF15 and *Agromyces aurantiacus* 95/TGF15 could utilize some of the toxic compounds present on TGF and use these as carbon sources [117]. An initial colonization of TGF by *C. ligniaria* F/TGF15 was crucial to allow establishment of selected bacteria on the TGF [116] *C. ligniaria* F/TGF15 thus paved the way for bacterial growth by removing bacteriostatic or bactericidal factors from TGF. Within the consortium of the aforementioned seven bacterial strains, four were well known as plant-health-promoting species [116]. Up to now, colonization in TGF was studied under otherwise sterile conditions in small systems, whereas in practice TGF will be applied for plant growth in larger systems. The volume needed for real-world situations will not allow expensive sterilization by gamma irradiation

and during incubation the moistened substrate will be in contact with (non-sterile) air. Information about the colonization of TGF by the selected strains under non-sterile conditions is not available yet.

The current experiment aimed to test the ability of the previously-selected ascomycete *Coniochaeta ligniaria* F/TGF15 and the plant-beneficial bacteria *Pseudomonas putida* 15/TGE5, *Serratia plymuthica* 23/TGE5, *Pseudomonas corrugata* 31/TGE5, *Methylobacterium radiotolerans* 56/TGF10, *Leifsonia xyli subsp. xyli* 66/TGF10, *Mycobacterium anthracenicum* 70/TGF15 and *Agromyces aurantiacus* 95/TGF15 to colonise TGF under non-sterile conditions. TGF with different inoculation treatments was applied in a potting mix, alone or mixed with peat, and evaluated in the greenhouse to test its suitability as a matrix for plant growth. Cultivation-based and DNA-based methods, i.e. PCR-DGGE and BOX-PCR, were applied to analyze the microbial and fungal communities in the matrix. The quality of TGF as a potting substrate was assessed by measuring the germination of tomato seeds, as well as plant weight and leaf area. In addition, the presence of phytotoxic compounds in the TGF substrate was assessed with GC-MS.

Material and Methods

Preparation of TGF

Grass material was collected in August 2004 from unfertilized, semi-natural grassland on a peaty clay soil near Wageningen, the Netherlands. The biomass was harvested as a first cut and consisted of a mixture of more than 30 species (mainly grasses, rushes, sedges and herbs) with a dry matter content of 37.5 %. Dry matter content of the plant biomass was measured after drying aliquots at 105^{o}C. The harvested material was dried at 105^{o}C, ground in a hammer mill to pieces <1 mm and stored until use in torrefaction experiments.

The ground material was torrefied in a preheated Carbolite muffle furnace (Model CWF 1100) at a temperature of 240^{o}C during 1 h. To ensure the occurrence of anoxic conditions, ground biomass was placed in aluminum boxes (approximately 40 g dry matter in a 200 ml box). The boxes were closed with their lid leaving a narrow split for gas exchange. The boxes were placed upside down in the oven to promote homogeneous heating of the contents. Boxes were weighed before and after heating to determine weight loss of the samples. The loss of dry weight due to torrefaction was 11 %. The pH of the TGF was 5.2. TGF was stored air-dry at room temperature in a closed plastic container.

Preparation of a fungal inoculum

The fungal strain *Coniochaeta ligniaria* F/TGF15 was grown on potato dextrose agar (PDA; 39 g/l, Oxoid, London, UK) for 10-12 days at 20 ^{o}C in the dark. Then, 10 ml

sterilized tap water was added per plate and fungal mycelium and spores were scraped off with a spatula. The optical density (OD_{600}) of the suspension was adjusted to 0.1 with sterilized tap water and measured using a Beckman DU® 530 Life Science UV/Vis spectrophotometer. The suspension was further diluted 1000-fold to obtain approximately 10^5 fungal propagules per ml suspension. Inoculum concentrations were enumerated by plating on R2A (Difco, Detroit, MI, USA).

Preparation of a bacterial inoculum

Freshly-grown bacterial cells of *Pseudomonas putida* 15/TGE5, *Serratia plymuthica* 23/TGE5, *Pseudomonas corrugata* 31/TGE5, *Methylobacterium radiotolerans* 56/TGF10, *Leifsonia xyli subsp. xyli* 66/TGF10, *Mycobacterium anthracenicum* 70/TGF15 and *Agromyces aurantiacus* 95/TGF15 were harvested from R2A (Difco, Detroit, MI, USA) by adding 5 ml mineral salt medium (MSM: K_2HPO_4 1.4 g/l, KH_2PO_4 1.4g/l, $MgSO_4.7H_2O$ 0.1 g/l, $(NH_4)_2SO_4$ 1.0 g/l, $MnSO_4.7H_2O$ 0.001 g/l, and $FeSO_4.7H_2O$ 0.01 g/l) to the Petri dishes. The optical density (OD_{600}) of the suspensions was adjusted to 0.1 with MSM and 1000-fold dilutions were made to obtain suspensions with approximately 10^5 CFU per ml. The inoculum concentrations were checked by plating on R2A.The bacterial consortium with seven strains in roughly equal CFU numbers was mixed with the TGF.

Colonization of TGF

In total, 12 spawn bags (30 x 60 cm) containing a 160-mm filter with 100-µm perforations (Van Leer Packaging, Pont-Audemer Cedex, France), were filled with 420 g air-dry TGF. Four treatments, each with three replicates, were prepared: C, uninoculated control, F, fungus alone, F+2B, the fungus plus bacterial strains *S. plymuthica* 23/TGE5 and *P. corrugata* 31/TGE5, and F+7B, the fungus plus seven bacterial strains, i.e. *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, *M. radiotolerans* 56/TGF10, *L. xyli subsp. xyli* 66/TGF10, *M. anthracenicum* 70/TGF15 and *A. aurantiacus* 95/TGF15. The *C. ligniaria* F/TGF15 inoculum was added to TGF, moisturizing TGF up to 50 % of its water holding capacity. In the control treatment, sterilized tap water was used for this purpose. The treated TGF was mixed well and incubated for 5 days at 25 ^oC in the dark. Subsequently, the bacterial inocula were added to treatments F+2B and F+7B using 10^5 cells of the bacterial mixtures per g dry TGF. Sterilized MSM was added to the control treatment as well as to treatment F. All treatments were moistened to 60 % of the TGF water holding capacity, mixed well and incubated for another 7 days at 25 ^oC in the dark.

Bacterial and fungal populations in the different treatments were analyzed over time by plate counts and PCR-DGGE. The pH and presence of selected compounds (GC analyses) were measured 12 and 26 days after the first inoculation. The four TGF

treatments incubated for 12 days were used, alone or mixed with peat, to test tomato seed germination and plant growth. The seed germination test was repeated with the TGF treatments when they were incubated during 26 days.

Assessment of microbial colonization

Bacterial and fungal populations in the four TGF treatments, each with three replicates, were enumerated by plate counts. A sample of 5 g moist TGF was shaken vigorously in 95 ml 0.1 % sodium pyrophosphate and 10 g gravel. Tenfold dilution series were prepared in 0.85% NaCl and plated on R2A medium. Results are presented as logarithmic numbers of CFU per g dry TGF. The introduced fungal strain *C. ligniaria* F/TGF15 was recognized by its typical morphological characteristics. The presence of the seven inoculated bacterial strains was detected by performing BOX-PCR on 100 randomly-selected colonies from the F+7B treatment after 26 days incubation.

BOX-PCR fingerprints of the colonies were performed by PCR with primer BOX-261 (5' - CTA CGG CAA GGC GAC GCT GAC G- 3'). Briefly, 1 µl of cell lysate was added to 24 µl of a mix containing 15 µl dNTP (Boehringer, Almere, the Netherlands), 2.5 µl buffer super Taq, 0.25 µl primer, 3.75 µl $MgCl_2$ (Perkin-Elmer, Nieuwersluis, the Netherlands), 0.25 µl superTaq and 2.75 µl MQ. Thermal cycling was as follows: initial denaturation at 95 ^{o}C for 7 min, followed by 90 ^{o}C for 30 s, 95 ^{o}C for 1 min, 52 ^{o}C for 1 min, 65 ^{o}C for 8 min, 29 cycles of 90 ^{o}C for 30 s and 65 ^{o}C for 16 min. The PCR products were analyzed by running 25 µl aliquots of the reaction mixtures in 1.5 % agarose gel in 0.5 X TBE buffer for 3h, 25 mV. Strains that were not successfully amplified with the BOX 261 primer were subjected to a second PCR reaction in which a diluted volume of crude cell lysate was added.

Bacterial and fungal PCR-DGGE analyses

Total community DNA of the four treatments were extracted and purified with the UltraClean^{TM} soil DNA kit (MoBio Laboratories, BIOzymTC, Landgraaf, the Netherlands) according to the protocol of the supplier, except that the cells were disrupted by bead beating 2 times 30 s in a Braun's cell homogenizer (Braun, Melsungen, Germany) at maximum speed. For disruption, glass beads (50 mg, 0.11 mm dia) were added to 0.5 g of TGF. The bead beating step was included to ensure maximal cell lysis without severe shearing of the DNA. DNA quality and quantity were assessed by electrophoresis in 1 % agarose gels in 0.5 x TBE buffer [103] by comparison to a standard 1-kb ladder (Invitrogen, Cat.15615-024, Carlsbad, USA). DNA size was 10-40 kb, on average.

Amplification of 16S ribosomal (r) RNA genes was done by using PCR in 50-µl reaction volumes as described before. For DGGE, a GC-clamped version of U968, i.e. U968-GC(5'-

CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAAC CTTAC-3') was used. Thermal cycling was as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 65°C for 90 s, 72°C for 2 min, final extension at 72°C for 10 min. The PCR products (expected sizes about 450 bp) were analyzed by running 5 µl aliquots of the reaction mixtures in 1 % agarose gels.

For fungi, a nested PCR of the ITS region was performed. PCR-ITS 1 was carried out in 25-µl reaction volumes containing 0.4 µM of each primer, 0.5 mM $MgCl_2$ (Perkin-Elmer, Nieuwersluis, the Netherlands) and 200 µM of each dNTP (Boehringer, Almere, the Netherlands) and 0.25 µg of T4 gene 32 protein (Boehringer, Mannheim, Germany) using 3.5 U expand HF polymerase in HF buffer 2. The primers used were ITS4, ITS primer in 28S (TCC TCC GCT TAT TGA TAT GC) and EF4, ITS primer, located in 18S rDNA (GGA AGG GRT GTA TTT ATT AG). The PCR product was used as a template for PCR-ITS2. PCR-ITS2 was carried out in 50-µl reaction volumes containing 0.4 µM of each primer, 0.5 mM $MgCl_2$ (Perkin-Elmer, Nieuwersluis, the Netherlands) and 200 µM of each dNTP (Boehringer, Almere, the Netherlands) and 5 U Super Taq polymerase in super Taq Buffer. The primers used were ITS2 primer in 5,8S (GCT GCG TTC TTC ATC GAT GC) and ITS1F primer +GC -clamped version in 18S (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCT TGG TCA TTT AGA GGA AGT AA).

Bacterial DGGE analyses were done using 6% polyacrylamide gels (ratio of acrylamide to bisacrylamide 37:1) with a gradient of 45 % to 65 % denaturants (100 % denaturant was defined as 7 M urea plus 40% formamide). The gradient of fungal DGGE was 30% to 80% denaturants. The gels were run at 60 ^{0}C (100 V) for 16 h in a Phor-U2 apparatus (Ingeny International, Goes, the Netherlands) and stained with SYBR gold (Molecular Probes, Leiden, the Netherlands). For analysis of the molecular community profiles, the Molecular Analyst fingerprint software (version 6.0, Biorad, Veenendaal, the Netherlands) was used.

GC MS analyses and pH

Extracts of the four different TGF treatments, each with three replicates, were prepared by the following protocol: 4.3 g of torrefied grass fiber was cold-extracted at room temperature with 113 ml distilled water for 2 h using a magnetic stirrer at 700 rpm. Following the cold extraction, the suspension was paper-filtered to remove grass fiber particles. The extract was then filter-sterilized (Millex GV bacterial filter Unit 0.22 µm Millipore) and GC MS analyses were done. Two ml filtrate and 2 ml of dichloromethane were mixed and the mixture was vortexed for about 10 sec, until a homogeneous solution was obtained. After centrifugation (1,200 x g, 5 min), the upper inorganic layer was discarded and the organic layer was transferred to a clean vial and passed over a short column (Pasteur capillary pipette filled with siliconized glass wool and anhydrous Na_2SO_4).

After this, the clean filtrate was collected and stored at -20^0 C until measurements were performed. Samples were concentrated 5-fold under a gentle flow of nitrogen gas.

Two µl of the filtered organic phase were injected in splitless mode into the injection port of a gas chromatograph (5888 series II, Hewlett-Packard GMI, USA) coupled to a mass spectrometer (model 5972A, Hewlett-Packard GMI, USA) with a Zebron ZB-5ms column (30 m × 0.25 mm I.D. × 0.25 µm film thickness) (PheNMenex, USA). The carrier gas was He (1ml min^{-1}). The oven was programmed at an initial temperature of 45°C for 1 min, with a ramp of 10°C per min to 310°C, and final time of 8.5 min. The injection temperature was 250°C and the detection temperature was 290°C. Peaks were tentatively identified by comparison of the spectra to commercial databases as well as to reference compounds: phenol (QBiogene, USA), pyrrole-2-carboxaldehyde, 2-methoxyphenol, 2,6-dimethoxyphenol, 2-furaldehyde and furan-2-methanol (Alfa Aesar GmbH & Co.KG, Germany).

The pH of the four colonization treatments in TGF after 12 and 26 days incubation was measured in the solid substrate by placing an electrode directly into the moist TGF.

Plant assay

TGF of the four colonization treatments after 12 days incubation were mixed in different ratio's with a non-fertilized horizontally harvested peat (P) from the Baltic region (Lentse Potgrond, Cuijk, the Netherlands). The pH of the peat was 3.5. Mixtures of 100%:0% 50:50 and 20:80 and 0%:100% TGF:P were used. Plastic pots 6 x 6 x 6 cm were filled with 200 g of the mixed substrates. Nine tomato seeds (cv Pronto, De Ruiter, the Netherlands) were sown per pot. All treatments had 6 replicate pots in a block design. The pots were watered after sowing and covered with plastic for a week. Thereafter, water was given 2-3 times a week and the nutrient solution once weekly. This nutrient solution contained 0.5 NH_4, 7.2 K, 0.5 NA, 3.1 Ca,1.6 Mg, 9.4 NO_3, 0.3 Cl, 2.8 S, 0.5 HCO_3 1.34 P, 0.01 Si [mmol/l] and 35 Fe, 3.5 Mn, 4.3 Zn, 25 B, 0.7 Cu and 0.6 Mo [µmol/l], pH 5.9, EC 2.0 [mS/cm]. The plants were cultivated in the greenhouse with 16 h light / 8 h dark (at 20 ^{o}C) day/night at 70 % relative air humidity. Seed germination was scored 7 and 14 days after sowing.

After assessment of germination, one healthy plant per pot was maintained and the surplus plants were removed. The single plant per pot was harvested 14 days later; plant weight (sprout) and area of detached leaves were measured.

The germination assay was repeated with the four colonization treatments of TGF which had been incubated for 26 days.

Statistical analysis

Analyses of variance (ANOVA) were performed for all measurements with the statistical program Genstat 10 (Rothamsted Experimental Station, Harpenden, UK). Least significant differences (LSD) were calculated at a significant level of P=0.05. CFU were analyzed after logarithmic transformation. Standard deviations were calculated for the GC data.

Results

Bacterial populations

Bacterial populations increased from the introduced 10^5 CFU/g TGF up to 10^8 CFU/g after 12 days and nearly 10^9 CFU/g after 26 and 40 days of incubation in the inoculated treatments (Fig. 1).

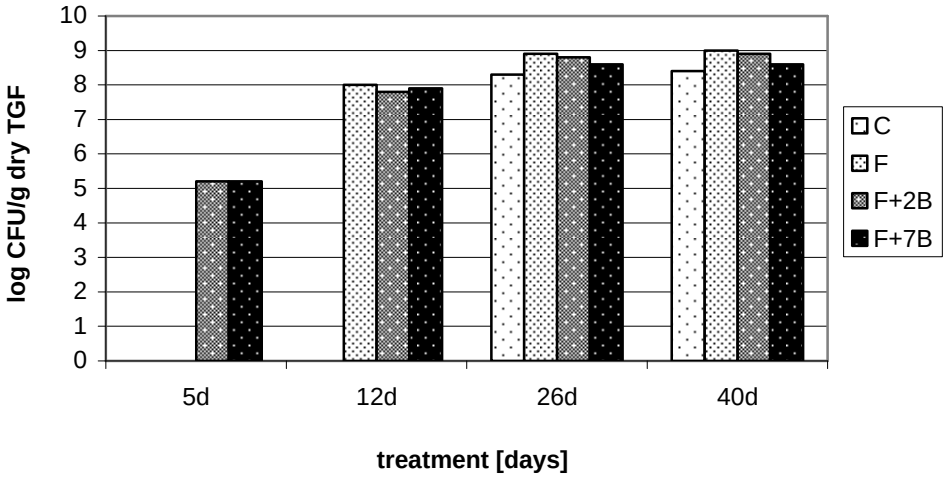


Fig. 1 Total cultural bacterial population in TGF on 12, 26 and 40 days after fungal inoculation. Treatments are: C, control without inoculants; F, *C. ligniaria* F/TGF15; F+2B, *C. ligniaria* F/TGF15, and 5 days later *P. putida* 15/TGE5 and *S. plymuthica* 23/TGE5; F+7B, *C. ligniaria* F/TGF15, and 5 days later 7 selected bacteria. LSD = 0.47.

In the treatment F+2B, two colony types dominated, i.e. red colonies corresponding to *S. plymuthica* 23/TGE5 and white slimy colonies corresponding to *P. corrugata* 31/TGE5. The percentages of *S. plymuthica* 23/TGE5 CFU were 37, 31 and 12 % in the treatment F+2B after 12, 26 and 40 days, whereas *P. corrugata* 31/TGE5 encompassed the remainder. *S. plymuthica* 23/TGE5 CFU made up 14, 17 and 11 % in the F+7B treatment after 12, 26 and 40 days incubation, respectively. In the latter treatment after 26 days, all seven inoculated bacterial strains were found back by BOX-PCR using 100 randomly selected colonies. The occurrence of strains was: 15% *P. putida* 15/TGE5, 17% *S. plymuthica* 23/TGE5, 18% *P. corrugata* 31/TGE5, 8% *M. radiotolerans* 56/TGF10, 12% *L. xyli subsp. xyli* 66/TGF10, 12% *M. anthracenicum* 70/TGF15, and 13% *A. aurantiacus* 95/TGF15. Only 5% of the colonies did not correspond to the seven introduced strains, showing a clear dominance of these.

The systems which had not been inoculated with any bacterial strains contained or acquired indigenous bacterial populations. These populations increased up to 10^9 CFU/g in treatment F and 5 x 10^8 CFU in the uninoculated control after 26 and 40 days (Fig. 1).

The DGGE fingerprints of the treated TGF on day 12 (Fig. 2) showed a dominant band corresponding with the introduced *S. plymuthica* 23/TGE5 and a double band representing *P. corrugata* 31/TGE5 in treatment F+2B. Treatment F+7B contained, additionally, the bands of *L. xyli subsp. xyli* 66/TGF10 and *M. anthracenicum* 70/TGF15 and probably of *A. aurantiacus* 95/TGF15. *P. putida* 15/TGE5 and *M. radiotolerans* were not detectable on gel since their bands overlap with those of *P. corrugata* 31/TGE5. The introduced *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, *L. xyli subsp. xyli* 66/TGF10, *M. anthracenicum* 70/TGF15 and *A. aurantiacus* 95/TGF15 were also detectable in the DGGE patterns at 26 and 40 days of incubation (data not shown).

From control treatment C, in which no bacteria had been introduced, no bacterial DNA could be obtained on day 12 and it was therefore not included in the DGGE fingerprint (Fig. 2). Treatment F revealed several bands which were different from those of the introduced strains. The absence of DNA in treatment C and the presence of bacterial bands in treatment F (Fig. 2) corresponded with the absence and presence of bacterial CFU on day 12 (Fig. 1).

Fungal populations

Total fungal populations were 10^7 to 10^8 CFU/ g TGF on days 12, 26 and 40 (Fig. 3). The fungus *C. ligniaria* F/TGF15 dominated (>95 %) in all treatments where it was introduced (data not shown). In treatment C it was not used. In this treatment, a pink-orange fungus abundantly colonized the TGF after 12 days. Growth of this fungus was consistently absent in all systems into which *C. ligniaria* F/TGF15 was introduced.

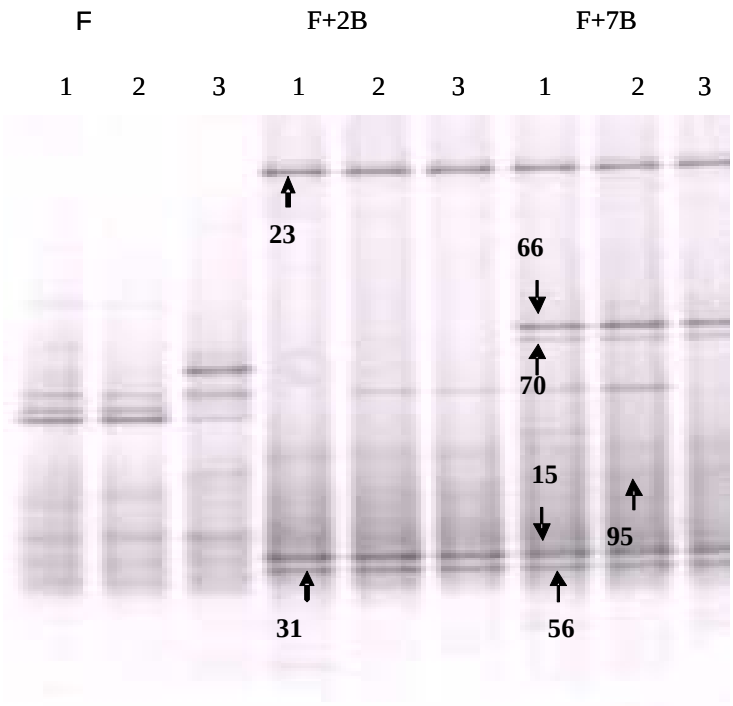


Fig. 2 Bacterial DGGE fingerprint of TGF on day 12. Treatments are: C, control without inoculants; F, *C. ligniaria* F/TGF15; F+2B, *C. ligniaria* F/TGF15, and 5 days later *P. putida* 15/TGE5 and *S. plymuthica* 23/TGE5; F+7B, *C. ligniaria* F/TGF15, and 5 days later 7 selected bacteria. Numbers 15, 23, 31, 56, 66, 70 and 95 correspond with the band of *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, *M. radiotolerans* 56/TGF10, *L. xyli subsp. xyli* 66/TGF10, *M. anthracenicum* 70/TGF15, and *A. aurantiacus* 95/TGF15, respectively. The band of 15 is similar with the upper band of 31, the band of 56 is similar with the lower band of 31.

C. ligniaria F/TGF15 was clearly detected in the fungal DGGE patterns of treatments F, F+2B and F+7B on days 12, 26 and 40 (data not shown). The numbers of other bands in the DGGE patterns from these treatments was limited, i.e. ranged from 4 to 7. In general, larger numbers of bands (i.e. 5-7) occurred at day 26 and 40 compared to day 12 (i.e. 4-5).

GC MS analyses

In total, four compounds were detected with GC analyses (Fig. 4) out of 15 target compounds which were previously found in the extract of TGF [117]. These four compounds were phenol, 2-methoxyphenol, 2-one-benzopyran, and tetrahydro-5,6,7,7a-

benzofuranone. In general, large variation between the levels of these compounds in independent samples was detected, resulting in large LSD values.

The measurable levels of the four compounds were low in treatment F+7B on day 12 compared to those in the other treatments. In fact, this treatment (the fungus in combination with the seven selected bacteria) showed most decreased (lowest) values in all four compounds after 12 days incubation. Furthermore, compound levels decreased significantly during the incubation period and, on day 26, all compounds were depleted or hardly detectable (Fig 4).

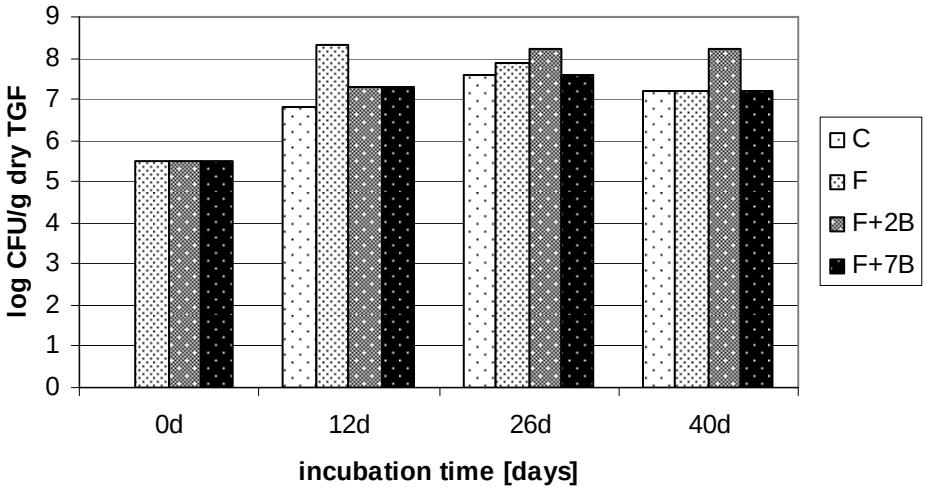
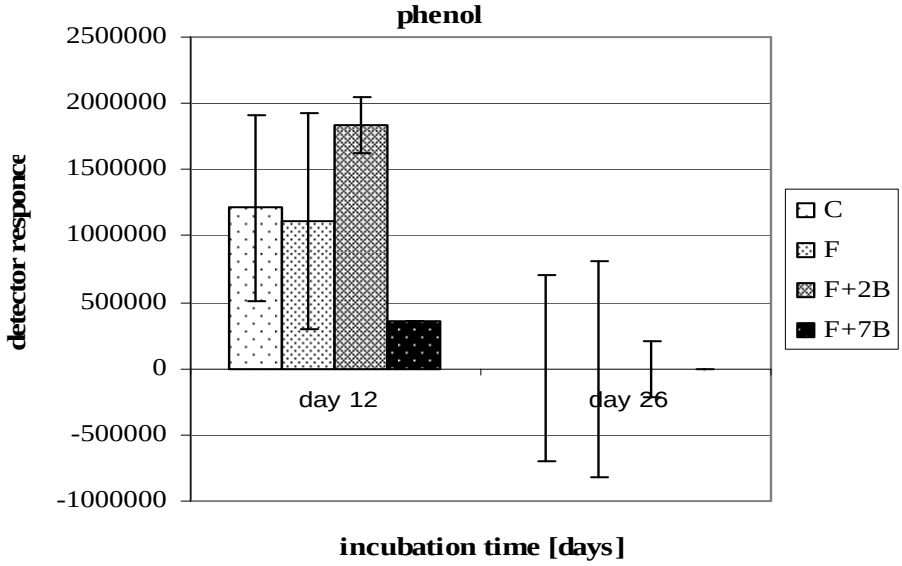


Fig. 3 Total culturable fungal population in TGF on 12, 26 and 40 days after inoculation. Treatments are: C, control without inoculants; F, *C. ligniaria* F/TGF15; F+2B, *C. ligniaria* F/TGF15, and 5 days later *P. putida* 15/TGE5 and *S. plymuthica* 23/TGE5; F+7B, *C. ligniaria* F/TGF15, and 5 days later 7 selected bacteria. LSD = 0.56.

pH

The pH values in TGF increased remarkably in all treatments colonized with the fungus *C. ligniaria* F/TGF15; from 5.2 in the fresh TGF up to 7.5 after colonization (Table 1). The pH in the control treatment (without inoculation) was 6.5 and 6.8 on day 12 and 26, respectively. Especially in the control treatment, the variation between the replicates was large.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2000000
1500000
1000000
500000
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detector responce
day 12
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C
F
F+2B
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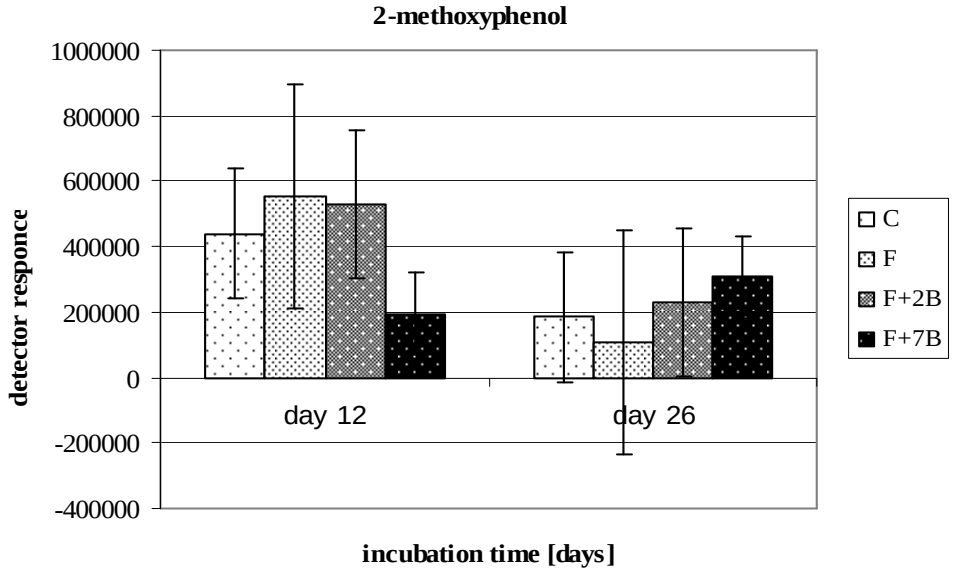
incubation time [days]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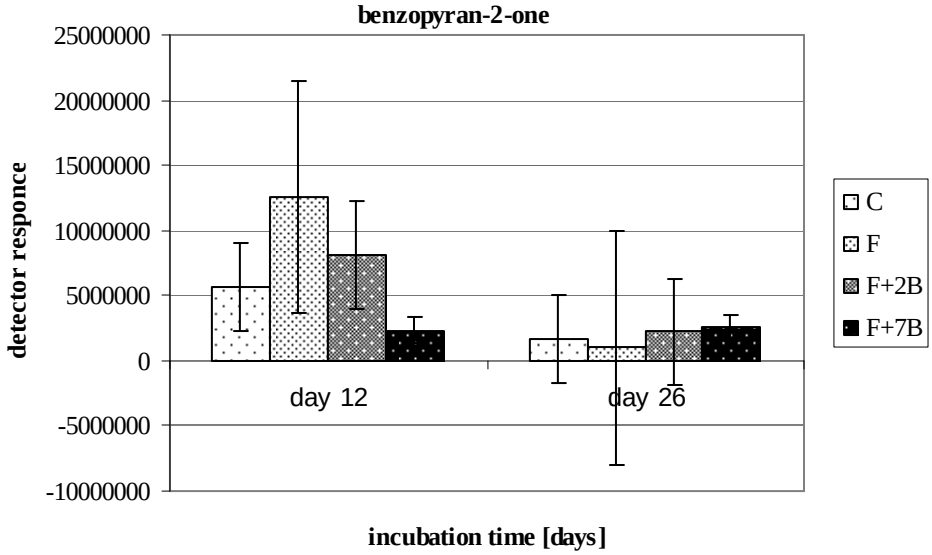
incubation time [days]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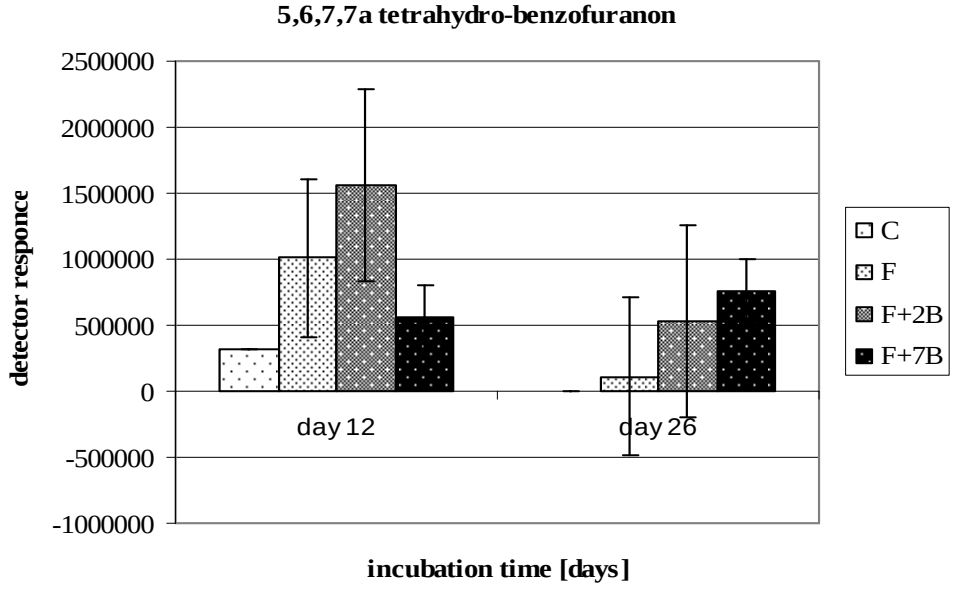
incubation time [days]


5,6,7,7a tetrahydro-benzofuranon
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C
F
F+2B
F+7B
incubation time [days]

Fig. 4 Presence of phenol, 2-methoxyphenol, 2-one-benzopyran, and tetrahydro-5,6,7,7a-benzofuranone in TGF 12 and 26 days after inoculation measured with GC analyses. Treatments are: C, control without inoculants; F, *C. ligniaria* F/TGF15; F+2B, *C. ligniaria* F/TGF15, and 5 days later *P. putida* 15/TGE5 and *S. plymuthica* 23/TGE5; F+7B, *C. ligniaria* F/TGF15, and 5 days later 7 selected bacteria. LSD values are respectively: 996910, 334214, 6408726 and 565301.

Plant assay

Seed germination was assessed 7 and 14 days after sowing into the pots containing the differently treated potting mixes (fig 5a and 5b). The results of 12 days colonized TGF are shown in Fig. 5.

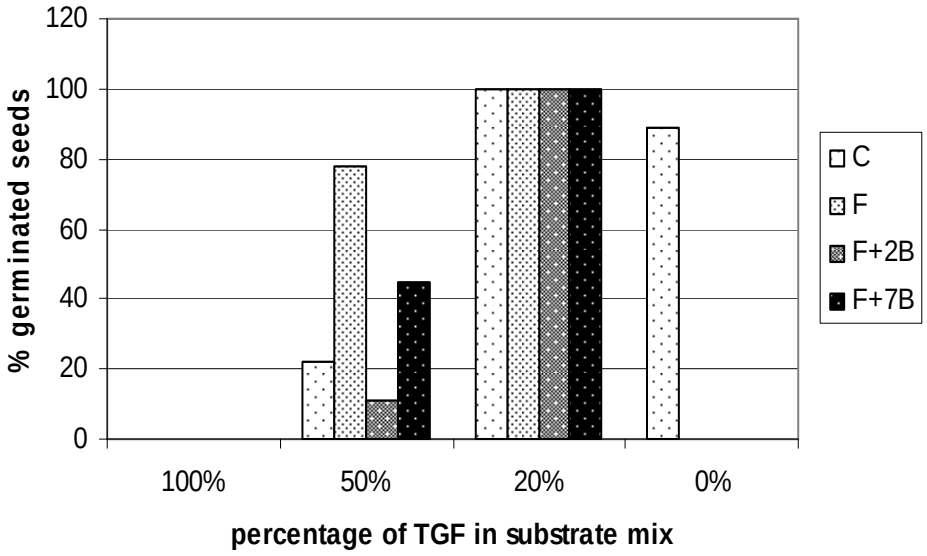

Fig 5a Percentage of germinated tomato seeds after 7 days and 14 days (fig 5b) in different TGF-peat mixtures. Treatments are: C, control without inoculants; F, *C. ligniaria* F/TGF15; F+2B, *C. ligniaria* F/TGF15, and 5 days later *P. putida* 15/TGE5 and *S. plymuthica* 23/TGE5; F+7B, *C. ligniaria* F/TGF15, and 5 days later 7 selected bacteria. LSD values are 13.4 and 15.3, 7 and 14 days after sowing respectively.

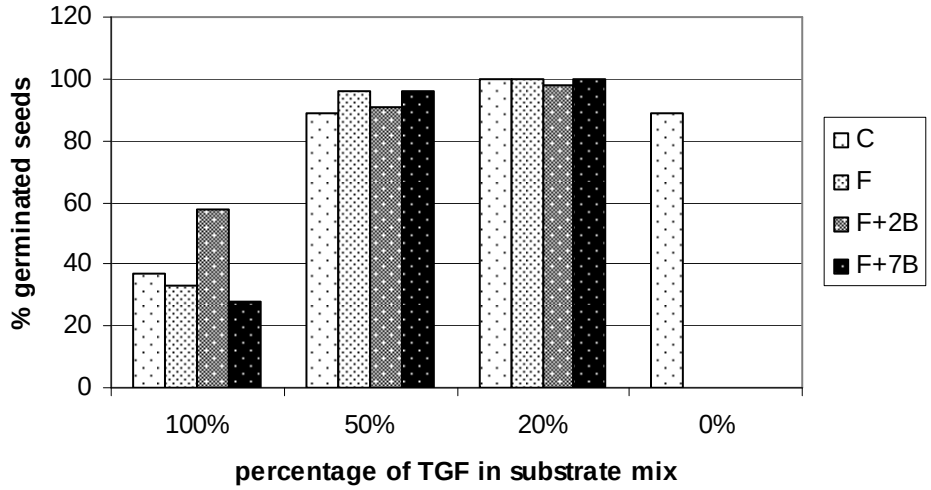

Fig. 5b

Germination of seeds in pure TGF (100 %) was 0 and 30-60 % after 7 and 14 days, respectively. Germination in 50 % TGF was 10-80 % after 7 days, but increased up to 90-95 % after 14 days. All seeds had germinated after 7 days in 20 % TGF, and hence this matrix was the best substrate for tomato seed germination among the tested TGF-peat mixtures. The pure peat (0 % TGF) showed high, but incomplete, germination rates, i.e. 90 % on days 7 and 14. Contrary to the expectation, none of the inoculum treatments gave a significant improvement of seed germination in the 50 and 20 % TGF substrate mix. Only in 100 % TGF, treatment F+2B resulted in an increased germination after 14 days compared to the control treatment C.

Seed germination in the different TGF treatments after 26 days colonization had similar results and equal levels of germination as in the first test with 12 days colonized TGF (data not shown).

Values of plant weight are shown in Fig 6. Plant weight in treatment C was low when using 100 % TGF, higher with 50 % and highest with 20 % TGF. Plant weight was extremely low (0.1 g) in the pure peat (0 %). Significant improvement was found in the treatments in which the fungus *C. ligniaria* F/TGF15 was used. Especially in the mixture of 50 % TGF with 50 % peat, plant weight doubled in treatments with the fungus as compared to that in the control. When the fungus was present, the mixture with 50 % TGF was as good as the mixture with 20 % in stimulating plant growth. Similar results were found for the treatments F+2B and F+7B, which were significantly better than the control treatment C in both, 50 % TGF and 20 % TGF. The co-presence of bacterial inoculants also had some

effect in 100 % TGF, showing a slight increase (not significant) in plant weight in treatment F+2B and F+7B compared to treatment C and F.

The results of the plant weight were confirmed by the leaf area measurements (Fig 7). Again, *C. ligniaria* F/TGF15 (treatment F) either or not in combination with two (treatment F+2B) or seven bacterial inoculants (treatment F+7B) incited a larger leaf area than that observed in the control treatment C. And a slight improvement occurred in 100 % TGF with treatment F+2B and F+7B compared to treatment C and F.

Both the potting mixtures with 50 % and 20 % TGF showed good plant growth, i.e. high weight and large leaf area, when the fungus was present. The co-presence of bacterial inoculants hardly affected plant growth , however might be of utmost importance in cases when suppressible phytopathogens invade the matrix.

Discussion

This study is a continuation of our previous research where a microbial community including *C. ligniaria* F/TGF15 and several selected plant-beneficial bacteria were established on TGF [116]. The beneficial microbial consortium was selected on the

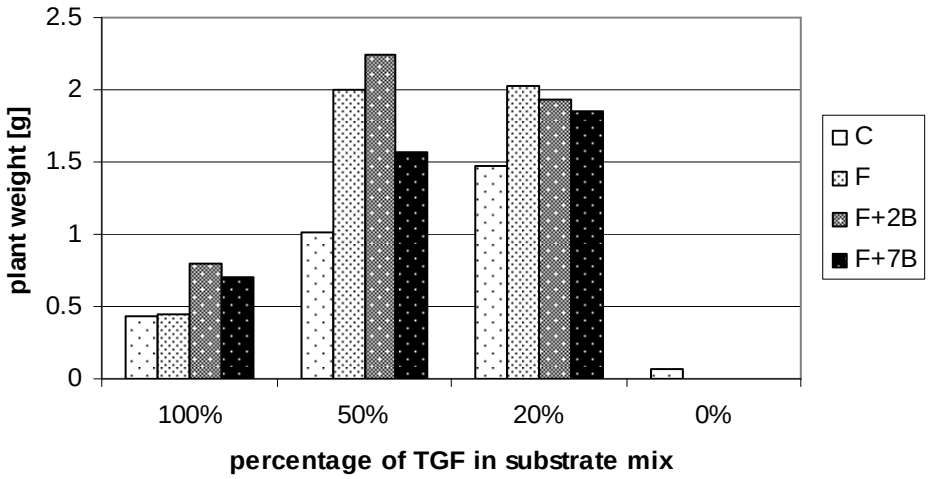

Fig. 6 Plant weight [g] of tomato plants 28 days after sowing. Treatments are: C, control without inoculants; F, *C. ligniaria* F/TGF15; F+2B, *C. ligniaria* F/TGF15, and 5 days later *P. putida* 15/TGE5 and *S. plymuthica* 23/TGE5; F+7B, *C. ligniaria* F/TGF15, and 5 days later 7 selected bacteria. LSD = 0.4.

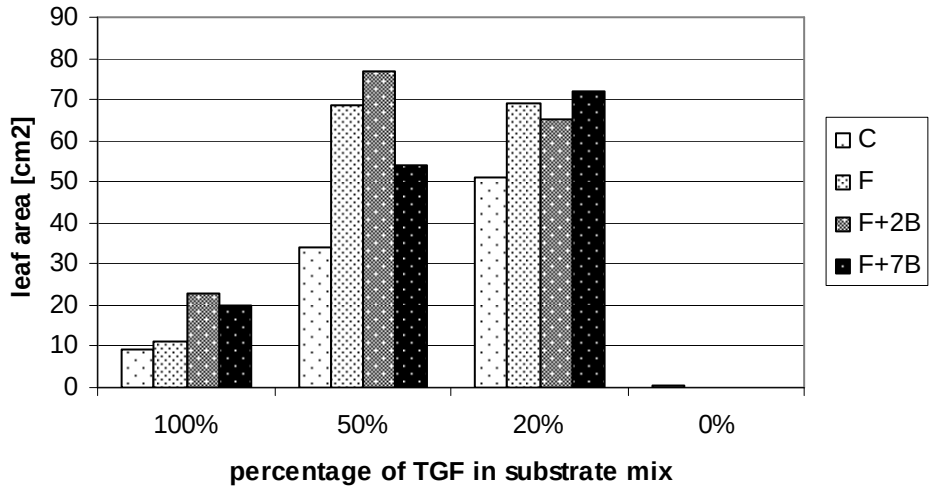

Fig. 7 Leaf area [cm^2] of tomato plants 28 days after sowing. Treatments are: C, control without inoculants; F, *C. ligniaria* F/TGF15; F+2B, *C. ligniaria* F/TGF15, and 5 days later *P. putida* 15/TGE5 and *S. plymuthica* 23/TGE5; F+7B, *C. ligniaria* F/TGF15, and 5 days later 7 selected bacteria. LSD = 15.8

basis of several parameters: (i) successful colonization and persistence on TGF, (ii) reduction of phytotoxicity in TGF extracts and (iii) the possession of plant-beneficial properties. In the current study, the practical application of TGF was evaluated in a greenhouse experiment with tomato seedlings, aiming to test the suitability of TGF as a peat substitute.

According to the CFU enumerations, the bacterial consortia of respectively two or seven bacterial species in combination with the fungus *C. ligniaria* F/TGF15 were relatively stable in the matrix during a period of 40 days. Populations reached nearly 10^9 CFU/g TGF. Since TGF was not incubated under sterile conditions, other bacteria were also found in some of the samples. However, the introduced bacterial consortia dominated and all introduced bacteria were found back with BOX-PCR among the isolated colonies after 26 days, being 95 % of the total culturable bacterial population.

Since the bags containing the different TGF treatments were exposed to open air, the control treatment (without microbial inoculants) was colonized by several bacteria plus an unknown pink-orange colored fungus. Uncontrolled colonization of potting mix is quite undesirable, especially if fungal mycelium is visually present [21]. Reassuringly, the pink-orange fungus was not found in treatments in which *C. ligniaria* F/TGF15 had been used. Thus, controlled inoculation of TGF with *C. ligniaria* F/TGF15 has the advantage that the substrate is prevented from undesirable and uncontrolled colonization by other fungi.

GC analyses revealed the presence of four compounds, phenol, 2-methoxyphenol, 2-one-benzopyran, and tetrahydro-5,6,7,7a-benzofuranone, in the TGF. A standard curve was prepared for 2-methoxyphenol [117], indicating that approximately 3 mg of this compound was present per kg dry TGF on day 12, which was roughly 30 % of the measured values in freshly torrefied grass. After 12 days incubation, the treatment with the fungus in combination with the seven selected bacteria had the lowest values for several of the compounds. The concentrations of all compounds decreased during the incubation period, and they were all depleted or hardly detectable after 26 days.

However, there was no clear reduction in the concentration of the compounds due to the introduced fungus *C. ligniaria* F/TGF15, in contrast to previous research where the fungus effectively removed several compounds, including phenol, from the extract of TGF [117]. The difference between the current and previous research was that respectively TGF and the extract of TGF were assessed. The discrepancy could be due to the fact that only part of the compounds (quantitatively and qualitatively) in TGF dissolve in the extract.

Regarding seed germination, the potting mix containing 20 % TGF was the substrate that allowed the highest germination rate: 100 % of the seeds germinated within 7 days. Pure TGF was apparently still too phytotoxic, with maximally 60 % germination after 14 days. Germination in the mix with 50 % TGF was delayed, however became substantial (90 % germination) over time. Unexpectedly, the seed germination was similar for the 12 and 26 days incubated TGF substrate. An increased germination was expected over time, since the compounds measured with GC-MS analyses decreased over time.

Unfortunately, seed germination was not improved by any of the bacterial inoculants or by the fungus. These inoculants had been selected, since previous studies showed that *C. ligniaria* F/TGF15 and the selected bacteria could reduce the toxicity and improve seed germination in extracts of TGF [117]. The fungus and several of the bacterial strains could grow on single model toxic compounds and reduce the presence of these compounds in the TGF extract [117]. The difference in results between the extract of TGF (previous results) and the solid TGF substrate again indicates that the solid substrate might contain more or other toxic compounds than its extract.

Interestingly, *C. ligniaria* F/TGF15 improved plant growth in the 20 and 50 % TGF mixtures. Leaf area as well as plant weight were significantly enhanced when the fungus was introduced into TGF. As a result, the mixtures with 20 as well as 50 % TGF with the fungus showed the best plant growth.

C. ligniaria F/TGF15 also influenced the pH of TGF upon colonization; pH increased up to 7.6. In the TGF control treatment without fungus, the pH was on average 6.6 and always lower than in the presence of *C. ligniaria* F/TGF15. The pH increase can be explained by utilization of the acid compounds in TGF by *C. ligniaria* F/TGF15.

The bacterial strains influenced seed germination and plant growth only occasionally under the more toxic conditions. Improved plant performance due to co-inoculation with the two bacteria was shown for seed germination in 100 % TGF, and for plant weight and leaf area in 100 and 50 % TGF. Although the effect of the bacteria was minor, they colonized TGF as a co-inoculant of the fungus. The plant-beneficial and pathogen-inhibiting bacteria among the inoculated consortium proliferated and formed a dominant population in TGF up to at least 26 days. In several other studies, colonization by beneficial microorganisms of compost [67, 90] or soil [3, 36, 55] was found to be disappointing, since the introduced microorganisms did not survive sufficiently. Thus, the successfully colonized TGF offers a unique opportunity for disease suppression. Data on this aspect are not available yet, since the substrate mix was free of pathogens.

With the current greenhouse experiment we showed that TGF can form an acceptable component of potting soil. It can be applied to up to 20 % in a potting mix if tomato plants have to be grown from seeds, or up to 50 % if germinated seeds are used. The ascomycete *C. ligniaria* F/TGF15 improved the quality of TGF as a partial peat replacement in potting soil and prevented the substrate from run-away colonization by other fungi. TGF was well colonized by the potential plant-growth-promoting bacteria *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5 and *P. corrugata* 31/TGE5. The colonization of the microbiologically virgin TGF with a plant-health-improving microbial consortium offers additional advantages to create a disease-suppressive substrate, meanwhile replacing peat in potting soil.

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CHAPTER 7

Summary and concluding remarks

In this thesis thermally treated (torrefied) grass fibers (TGF), which are chemically stabilized by heating for 1 h at 240 ^{o}C under low oxygen, are proposed, in combination with a beneficial microbial consortium, as a novel substratum to be used in potting soil that allows the growth of healthy plants. During the heating process, compounds leading to (phyto)toxicity are generated in the TGF. The aim of this study was therefore to develop a microbial consortium that (1) will remove the phytotoxic compounds from the matrix, and (2) will provide otherwise beneficial conditions for plant growth. In the following sections, the research questions posed in the general introduction (**chapter 1**) are addressed and placed in a broader context, in order to highlight the main findings achieved in this thesis.

1. What type of community introduced on TGF establishes and develops stably over time?

To answer this question, TGF and a TGF extract (denoted TGE) were inoculated with a microbial community obtained from soil and, using sequential growth steps, particular organisms were enriched (**Chapter 2**). We aimed to select those microorganisms that are adapted to establishment and growth in the TGF matrix and/or its (carbonaceous) compounds. The bacterial numbers in TGF were shown to consistently increase, from each dilution back to about the presumed carrying capacity of the TGF, i.e. about 10^{9} -10^{10} / g dry matter. This observation indicated that TGF in principle offers conditions favorable to the outgrowth of particular microorganisms.

The subsequent phylogenetic analysis of the isolates that dominated in the final communities revealed the occurrence of 10 to 15 different bacterial types. The organisms predominantly found in the TGE enrichments were for the most part Gram-negative and *r*-strategist bacteria, whereas the ones isolated from TGF were mainly Gram-positive and *K*-strategist bacteria. These micro-organisms dominated most probably because they could utilize the easily available carbon sources in TGF and TGE - acetate and formate. These compounds can theoretically serve as nutrient and energy sources for many micro-organisms. The 111 isolated strains were grouped in 17 groups by BOX-PCR and representative strains from each group were tested for growth on the main readily-available carbon sources in TGF, acetate (465.6 mg/kg) and potassium formate (791 mg/kg). Only few strains could grow on minimal media supplied with acetate or formate as sole carbon sources, however all strains (except one) grew on the combination of both C-sources.

Given the fact that rather stable band patterns were found in the total bacterial communities after only few enrichment steps in both systems (12 in TGF and 10 in TGE), it is likely that the underlying communities stabilized following the initial enrichment steps. Thus, both in TGF and TGE, several bacterial types presumably were co-selected by the

substratum, indicating the existence of diverse niches in it. The differences in the composition of the microbial consortia selected in the two media were likely related to differences in the physical nature of both substrates, i.e. liquid TGE versus solid TGF. Among the isolates obtained, similar richness values (determined by the numbers of different bacterial types – evidenced from phylogenetic analyses) were found, i.e. nine in TGE and ten in TGF enrichments. This observation matched with the DGGE analyses including similarity in richness values.

Furthermore, virtually all DGGE bands found from the TGE enrichments were matched by isolates, whereas this matching was less for the TGF enrichments. We obtained strong evidence for the contention that, in particular, members of the γ-proteobacteria as well as of the Cytophaga-Flavobacterium-Bacteroides (CFB) group were selected by the TGE enrichments, whereas specific α-proteobacteria and actinobacteria were typically found in the TGF enrichments.

The collection of bacterial and (some) fungal isolates that were selected by TGF and TGE based on their good adaptation to these substrates and their ability to use carbon sources from them were used in further analyses. Thus we selected a range of isolates that are TGF "competent", and were established after ecological competence.

2. Can toxic compounds present in TGF be transformed by a biodegradative microflora?

Phytotoxicity and bacterial toxicity of the TGE was found to be concentration-dependent, since it decreased after dilution of the extract. Hence, we surmised that particular chemical compounds were the underlying factors and identified several in TGF. These were 2-furaldehyde (27.9 mg/kg), phenol (11.2 mg/kg), 2-methoxyphenol (10.8 mg/kg), 2,6-dimethoxyphenol (10.2 mg/kg) and furan-2-methanol (8.6 mg/kg) [91]. To assess whether such phytotoxic compounds could be removed from the matrix, 88 bacterial and one fungal strain(s) isolated from TGF or TGE (**chapter 2)**, were grown in TGE and then a lettuce seed germination assay was performed on the centrifuged and pellet removed TGE. The assay thus monitored the reduction of toxicity to lettuce seeds in TGE. Eleven of the 88 bacterial strains and the fungus significantly reduced phytotoxicity. The 11 bacteria belonged to the *Pseudomonadaceae, Burkholderiaceae, Enterobacteriaceae, Methylobacteriaceae, Flavobacteriaceae, Microbacteriaceae, Rhizobiaceae, Mycobacteriaceae* and *Xanthomonadaceae,* whereas the fungus was identified as a *Coniochaeta ligniaria.*

As the level of reduction of each phytotoxic compound in the TGE was unknown, additional experiments were performed, under which a growth assay on each compound

used as single carbon source, and a GC-MS assessment of the removal of particular compounds by growth of selected strains in TGE.

Overall, *Coniochaeta ligniaria* was found to be the most effective organism in improving lettuce seed germination, and it grew on each of the six toxic compounds. It also removed most of the phytotoxic compounds present in TGE, including phenol and 2-methoxyphenol, as shown by GC-MS analyses. Interestingly, *C. ligniaria* is known to play a role in the biological detoxification of lignocellulosic hydrolysates [70, 71].

Among the bacteria, the most promising bacterium for colonization of TGF, due to biodegradation, was the *Methylobacterium radiotolerans* 56/TGF10. It was most active in increasing seed germination, improving the germination with about 35%. This isolate removed 2-furaldehyde, 2-furancarboxaldehyde-5-methyl, pyrrole-2-carboxaldehyde, 5-acetoxymethyl-2-furaldehyde, ethanone1-(4-hydroxy-3-methoxy phenyl) and 5-acetylaminomethyl-4-amino-2-methyl pyrimidine. Four other bacteria, *Pseudomonas putida* 15/TGE5, *Serratia plymuthica* 23/TGE5, *Pseudomonas corrugata* 31/TGE5 and *Agromyces aurantiacus* 95/TGF15, could decrease the concentration of seven of the 14 distinguished compounds present in TGE.

Overall, we found five bacterial strains together with the fungus *C. ligniaria* that appeared promising as constituents of a microbial consortium for inoculation of TGF, as they were capable to remove or decrease concentrations of at least seven compounds that presumably caused phytotoxicity and thus significantly enhanced lettuce seed germination (**chapter 3**). Thus, a microbial community with the capacity to remove phytotoxic compounds from TGE, to colonize TGF, and the potential to promote plant growth or plant health was selected.

3. What types of ecological interactions take place between fungal and bacterial parts of the consortium?

The selected (**chapter 3**) microbial community consisted of mainly *r*-strategist bacteria. Since we aimed a TGF community consisting of almost equally spread *r*- as well as *K*- strategist bacteria, 2 extra *K*-strategists, i.e. *Leifsonia xyli subsp. xyli* 66/TGF10 and *Mycobacterium anthracenicum* 70/TGF15, were included in the consortium. Thus, the final consortium selected for TGF colonization consisted of one fungus (*Coniochaeta ligniaria* F/TGF15) and 3 *r*- and 4 *K*-strategist bacteria, i.e. *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5, *M. radiotolerans* 56/TGF, *L. xyli subsp. xyli* 66/TGF10, *M. anthracenicum* 70/TGF15, and *A. aurantiacus* 95/TGF15. These microorganisms have non-overlapping niches (presumably), and will therefore co-exist.

Surprisingly, TGF was found to be very recalcitrant to bacterial colonization, as none of the selected bacteria, not even the TGF isolates, either applied as single strains or

as combinations of bacterial strains, was able to colonize and persist in the matrix. Although, TGF isolates became dominant when selected from the soil mix, if there are put back in TGF they did not survive.

The addition of selected easily available nutrients or a surfactant to the TGF, or increasing pH up to 7, did not enhance the persistence of any of the bacterial inoculants in the TGF. We then surmised that the TGF might contain bacteriostatic or bacteriocidal factors of unknown nature that could possibly be removed by cold-water washing. Indeed, washing of the TGF allowed several strains, namely *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5 and *M. radiotolerans* 56/TGF10, to grow and establish on the TGF, and hence particular detrimental compounds might indeed have been present. In contrast to the bacterial isolates, the fungus *Coniochaeta ligniaria* was shown to consistently grow and persist on TGF (**chapter 4**), and hence we surmised this organism might be the missing link that explains the discrepancy between the TGF enrichment and the bacterial recolonization data.

Indeed, precolonization of TGF for 3 days or more with *C. ligniaria* was shown to pave the way for bacterial establishment on TGF. Specifically, after 3, 6 and 13 days of precolonization with the fungus, a bacterial consortium consisting of *P. putida* 15/TGE5, *S. plymuthica* 23/TGE5, *P. corrugata* 31/TGE5 *M. radiotolerans* 56/TGF10, *L. xyli subsp. xyli* 66/TGF10, *M. anthracenicum* 70/TGF15 and *A. aurantiacus* 95/TGF15 could grow in the TGF, from about log 5.8 up to about log 10 CFU per g TGF.

This major finding provides an indication for the role of *C. ligniaria* as a colonization helper organism, promoting bacterial growth on TGF. Several mechanisms can be involved in this colonization helper effect; we postulate the following interactions:

a. Removal of bactericidal or bacteriostatic compounds by the fungus (which could be the same as the phytotoxic compounds)

b. Modulation of local colonization conditions (sites for adherence, water film, etc)

c. Liberation of nutrients by leaky fungal cell walls or actively excreted exudates.

In **chapter 5** we particularly address the possible inhibitory or stimulatory effects of selected bacteria in the interaction with *C. ligniaria* F/TGF15.

The possibility that bacterial strains *P. putida* 15/TGE5, *P. corrugata* 31/TGE5, *M. radiotolerans* 56/TGF, *L. xyli subsp. xyli* 66/TGF10 and *A. aurantiacus* 95/TGF15, would grow on exudates provided by the fungus was tested. All strains except *M. radiotolerans* 56/TGF10 revealed considerable growth over time. Hence, during translocation on agar TGF, these bacteria might have been using nutrients released by *C. ligniaria.*

P. putida 15/TGE5 and *M. radiotolerans* 56/TGF10 were chosen as model strains for the investigation of possible bacterial translocation via the fungus *C. ligniaria* in the

TGF matrix. *P. putida* 15/TGE5 was translocated via *C. ligniaria* F/TGF15 on TGF, but not *M. radiotolerans* 56/TGF10. Thus, in the light of the rapid death of *P. putida* 15/TGE5 on TGF without added *C. ligniaria*, it is likely that the fungus incited substantial bacterial movement and concomitant growth in the substrate. *M. radiotolerans* 56/TGF10 cannot multiply on fungal hyphae, due to its inability to grow in fungal exudates, as shown in a liquid culture.

In conclusion, *C. ligniaria* is a crucial factor for the colonization and detoxification of TGF, meanwhile stimulating growth and translocation of a consortium of plant growth promoting bacteria.

4. Will plant-beneficial (plant-growth-promoting or antagonistic) bacteria colonize TGF and stably persist on it?

The four gram-negative bacterial strains (3 *r*- and 1 *K*- strategist bacteria) that persisted in the TGF after pre-colonization with *C. ligniaria* are potentially plant-beneficial (**chapter 4**). Two of the consortium strains, i.e. *S. plymuthica* 23/TGE5 and *P. corrugata* 31/TGE5, had *in vitro* antagonism towards several phytopathogenic fungi, i.e. *Rhizoctonia solani*, *Fusarium oxysporum* sp. *radicis lycopersici*, *Verticillium dahliae*, *Pythium aphanidermatum* and *Pythium ultimum*. No fungal plant pathogen inhibition was found with the strains *P. putida* 15/TGE5 and *M. radiotolerans* 56/TGF10, but bacteria from these species are regularly implied as plant-beneficial bacteria as they produce phytohormones and support plant growth or induce disease resistance in the plant [40, 57, 133].

Antagonism towards *C. ligniaria* was tested as well and it was found that *S. plymuthica* 23/TGE5 and *P. corrugata* 31/TGE5 inhibit or kill *C. ligniaria* F/TGF15. Therefore, when these two bacteria are colonized in TGF together with the fungus, the fungus will be destroyed by them. However, this is not of any problem, since *C. ligniaria* F/TGF15 is important in the first colonization stage, followed by the bacterial colonization. Since visual fungal hyphae are not recommended in potting mix (cosmetic factor), this finding is an additional advantage for practical applications, where in potting mix after trans-location of antagonistic bacteria, the growth of *C. ligniaria*, as well as other fungi, will be suppressed. The capacity of such antagonistic bacteria to colonize TGF and migrate in it, can enhance their role as plant-health beneficial microorganisms.

5. Does microbially-colonized TGF constitute a suitable matrix for plant growth and can it be used as a substitute for peat?

In **chapter 6** we approached the practical application and the quality of TGF in a greenhouse experiment with tomato seedlings. The aim was to test the suitability of TGF as

a peat substitute. For this reason it was mixed in different dosages with a commercial peat product.

According to the CFU enumerations, the bacterial community of respectively two or seven bacteria and the fungus *C. ligniaria* F/TGF15 in treatments F+2B and F+7B was stable during a period of 40 days. TGF was exposed to external bacteria, since it was not incubated under sterile conditions. Other bacteria then the introduced community were found indeed, however, the introduced bacterial community dominated over the wild bacteria. All seven bacteria were found back with BOX-PCR of isolated colonies from TGF after 26 days.

We found at least four plant-growth promoting bacteria that multiplied and survived well in TGF. In contrast, in most of the cases, colonization by beneficial microorganisms of compost [67, 90] or soil [3, 36, 55] was found to be disappointing, since the introduced microorganisms did not proliferate sufficiently. Thus our strategy of selecting bacteria that could grow on specific (phyto)toxic compounds present in TGF, followed by an introduction in a microbiologically virgin substratum, was shown to be successful.

Although, there was no clear improvement of seed germination due to *C. ligniaria* F/TGF15 or the bacteria, the measurements of tomato leaf area [cm^2] as well as the values of plant weight [g] clearly showed improvement in all 3 combinations compared to the control where no microorganisms were added. There was a very clear improvement due to *C. ligniaria* F/TGF15. The bacterial strains influenced seed germination and plant growth only occasionally under the more toxic conditions.

The mixtures with 20 as well as 50 % TGF with the fungus and additionally treated with *P. putida* 15/TGE5 and *S. plymuthica* 23/TGE5 showed the best plant growth, respectively the best option for practical application. The 100% TGF was too toxic, and the 0% TGF (only peat) was not effective.

In conclusion, in **chapter 6,** it was shown that TGF can form a stable, plant-healthy component of potting soil. It can be applied to up to 20 % in a potting mix if tomato plants have to be grown from seeds, or up to 50 % if germinated seeds are used. Although the effect of the bacteria was minor, they colonized TGF as a co-inoculant of the fungus. The ascomycete *C. ligniaria* F/TGF15 improved the quality of TGF as a partial peat replacement in potting soil and prevented the substrate from run-away colonization by other fungi.

Concluding remarks and outlook

This thesis describes the potential of microbially-matured TGF to serve plant growth in potting soil. Several research questions were explored and partial answers were obtained.

After some unexpected results, we could successfully select a consortium for TGF colonization consisting of *r*- and *K*- strategist bacterial strains and a fungus. TGF was only stably colonized by both *r*- and *K*- strategist bacteria after an initial colonization by the ascomycete *C. ligniaria* F/TGF15 which had the unique capacity to degrade toxic compounds, including phenols. This major finding provides an indication for the role of *C. ligniaria* as a colonization helper organism, promoting bacterial growth, e.g. by removing bacteriostatic or bactericidal compounds from TGF. Inoculating *C.ligniaria* in advance was a crucial step that allowed establishment of bacterial beneficials.

Bacteria that show antagonism towards phytopathogenic fungi could stably colonize TGF. These results prove the potential of TGF to create a substrate containing a plant beneficial microbial population. Moreover it was shown that the stable colonization by the introduced microbial population prevented TGF from external microorganism's invasion.

The scientific investigation of TGF colonization concerning ecological as well as practical aspects elucidated several scientific findings in this thesis:

- *C. ligniaria* F/TGF15 together with some bacteria were capable to remove toxic compounds in TGF and its extracts.
- Discovery of the unique role of the ascomycete *C. ligniaria* F/TGF15 as colonization helper for bacterial growth in TGF colonization.
- The data on bacterial-fungal interactions revealed the importance of *C. ligniaria* F/TGF15 as bacterial mediator in TGF: 1. detoxification by fungus makes substrate suitable for the bacterial inoculants, 2. growth and transport of the bacterial inoculants on the fungal hyphae.
- The selection procedure aiming for bacteria growing on the phytotoxic compounds present in TGF was a successful strategy, since a microbial consortium able to colonize TGF and persist on it, reduce the toxicity and have plant-beneficial properties was achieved.

The new findings also created subjects for further investigations. The successfully colonized TGF offers a unique opportunity to produce a substrate with disease suppression. We showed the ability of the selected plant beneficial micro-organisms to stably colonize TGF. However, data on disease suppression are not available yet, since the substrate mix

was free of pathogens. It is of practical interest for the future to investigate the disease suppressive potential of this new substrate against different plant diseases in varying crops.

From scientific point of view, further approach on *C. ligniaria* in interaction with *P. putida* or some of the other bacteria described in the thesis is of great scientific interest. Spatial and temporal aspects of the positive and negative interactions between the organisms will allow a better understanding of ecological principles in natural as well as created substrates. In situ (microscopically) studies on how the bacteria colonize the fungal-colonized TGF substratum might help to understand the complex interactions occurring in these matrixes.

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Summary

This thesis describes the selection of a beneficial microbial consortium able to colonize torrefied (thermally treated) grass fibers (TGF) to be used as a novel substratum for plant growth. During the heating process, compounds leading to (phyto)toxicity are generated in the TGF. The aim of this study was therefore to develop a microbial consortium that (1) removes the phytotoxic compounds from the matrix, and (2) provides otherwise beneficial conditions for plant growth.

After sequential enrichment, a consortium was selected for colonization of TGF consisting of *r*- and *K*-strategist bacterial strains and an ascomycete fungus. Several bacteria, i.e. *Pseudomonas putida*, *Pseudomonas corrugata*, *Serratia plymuthica*, *Agromyces aurantiacus*, and the fungus *Coniochaeta ligniaria* F/TGF15 could decrease the concentration of seven toxic compounds. Only the fungus had the unique capacity to degrade phenols. *C. ligniaria* was found to be essential for the stable colonization of TGF by the selected bacteria; besides removing bactericidal compounds from TGF, it provided compounds for bacterial growth and allowed translocation of bacterial cells through the substrate. Three of the selected bacteria were displaying antagonism towards phytopathogenic fungi, thus creating a unique opportunity to produce a substrate with potential for plant disease suppression.

Finally, the colonized TGF was successfully applied as a compound in a potting mix for the growth of tomato seedlings. These results proved the potential of TGF as a novel substratum, meanwhile containing a plant beneficial microbial population.

Samenvatting

Dit proefschrift beschrijft de selectie van een microbieel consortium voor de kolonisatie van thermisch verduurzaamde grasvezels als substraat voor plantengroei. Tijdens de verhitting van de grasvezels ontstaan fytotoxische stoffen. Doel van het huidige onderzoek is om een microbieel consortium to ontwikkelen dat (1) de fytotoxiciteit vermindert en (2) plantengroei en plantgezondheid stimuleert.

Na opeenvolgende verrijkingscultures, is een consortium geselecteerd bestaande uit r- en K-strategie bacteriën en één schimmel. Enkele bacteriën, nl. *Pseudomonas putida, Pseudomonas corrugata, Serratia plymuthica,*en *Agromyces aurantiacus,* en de schimmel *Coniochaeta ligniaria* F/TGF15 verlaagden de concentratie van zeven toxische stoffen. Alleen de schimmel had de unieke eigenschap om fenolen af te breken. De schimmel bleek bovendien essentieel voor de kolonisatie van de verduurzaamde grasvezels door de bacteriën; naast afbraak van bactericiden, leverde het stoffen waar de bacteriën op konden groeien en zorgde het voor verspreiding van bacteriën door het substraat. Drie van de geselecteerde bacteriën waren antagonistisch ten aanzien van diverse plantpathogene schimmels, waardoor een unieke mogelijkheid ontstaat om substraat te produceren met een plantenziektewerend vermogen.

Uiteindelijk is gekoloniseerd grasvezelsubstraat succesvol toegepast in een mengsel met veen voor het kweken van tomatenzaailingen. Deze resultaten tonen aan dat verduurzaamde grasvezels een geschikt nieuw substraat zijn, en tegelijkertijd een voor de plant nuttige microflora bevatten.

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About the author

Radoslava Dimitrova Trifonova was born on the 2nd of January 1976 in Montana, Bulgaria. In 1994, she completed her secondary education-gymnasium in Sofia, Bulgaria. In the same year, she started her MSc studies in Biotechnology at the University of Sofia "St. Kliment Ohridski", Faculty of Biology. In July 1999, she defended her master diploma entitled "Monitoring of microbial corrosion in water-cooling system of energy plant pipelines" in the Microbiology Department, Sofia University. Her diploma work was carried out under the supervision of Assoc. Prof. Dr. V. Groudeva. After her graduation, Radoslava worked for a couple of months in the Institute of Genetics, Bulgarian Academy of Science. In 2000, she started working on a fellowship project entitled "In situ bioremediation of soils and waters polluted with heavy metals" in the Department of Microbiology, Sofia University. One year later in 2001, Radoslava participated in a joint project with Siena University, Italy, where she worked for a period of 6 months. After Italy, she wrote a short, mini proposal and applied for an Austrian governmental fellowship for postgraduate students in Eastern Europe to carry out a research in Seibersdorf, Austria. Radoslava received the fellowship and during 2002-2003, she worked in Seibersdorf Res. GmbH, Div Environment & Life Science, Biotechnology under the supervision of Dr. Angela Sessitsch. In Seibersdorf, Radoslava worked on a project entitled "Characterization of bacterial communities associated with flowering plants of the Ni hyperaccumulator *Thlaspi goesingense*". She investigated endophitic and rhizosphere bacteria by using cultivation and cultivation-independent techniques (T-RFLP). She studied bacterial Ni tolerance and she was involved in the characterization of a beneficial plant-microbe interactions, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase and siderophore production.

In 2004, Radoslava started her PhD in Wageningen at Plant Research International. Her project entitled "Microbial maturation of novel substrates" was a cooperation with the Microbial Ecology Department, Centre for Evolutionary and Ecological studies of the University of Groningen, with Prof. dr. J.D van Elsas as promoter and Joeke Postma (Plant Research International) as a copromotor. The results from this project are summarised in the present thesis.

Publications

Trifonova R., Postma J., Ketelaars J.J.M.H., and van Elsas J.D. (**2008**)
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PhD courses	**Date**
• Basic statistic course	13-20 June 2005
• Soil-Plant-Microbe Interactions, Uppsala, Sweden, **oral presentation**	27 June - 8 July 2005
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Scientific meetings	
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• Soil pathogens and Soil Microbiology meeting , Lisse, NL	7 April 2005
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• ECOINT meeting, Wageningen, **oral presentation**	28 November 2005
• Annual Ph.D meeting, Research school Biodiversity, Wageningen, **oral presentation**	1 December 2005
• Soil born pathogens,Wageningen	30 March 2006
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• Haren, **oral presentation**	18 January 2007
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Congresses/Symposiums	
• IOBC symposium, Wageningen , NL "Multitrophic interactions in soil", **poster presentation**	6-8 June 2005
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